# UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

DEPARTAMENTO DE SANIDAD ANIMAL


## TESIS DOCTORAL

# PATRÓN EPIDEMIOLÓGICO Y NUEVOS MÉTODOS DE CONTROL DE LAS NOSEMOSIS DE *Apis Mellifera L.* EN ESPAÑA

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR


**Cristina Botías Talamantes**

Directores

Aránzazu Meana Mañes
Mariano Higes Pascual
Raquel Martín Hernández


**Madrid, 2013**

Universidad Complutense de Madrid
Facultad de Veterinaria
Departamento de Sanidad Animal

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El trabajo presentado en esta tesis se ha realizado en el Centro Agrario Regional de la Junta de Comunidades de Castilla La Mancha (CAR) y la Universidad Complutense de Madrid (UCM), bajo la dirección de los Doctores Raquel Martín Hernández, Mariano Higes Pascual y Aránzazu Meana Mañes, como parte de los proyectos API 06-009, API 06-001, RTA2005-00152-00-00. Asimismo, la autora ha disfrutado de una beca predoctoral de la Junta de Comunidades de Castilla La Mancha (Orden de 18/12/2006 de la Consejería Agricultura de la JCCM). Durante el desarrollo de la misma se han realizado estancias en el Commonwealth Scientific and Industrial Research Organisation (CSIRO) gracias a las ayudas José Castillejo de la JCCM y en el Insitute National de la Recherche Agronomique (INRA) a través de las ayudas europeas COST Action FA0308.

ARÁNZAZU MEANA MAÑES, MARIANO HIGES PASCUAL Y RAQUEL MARTÍN HERNÁNDEZ, doctores en Veterinaria.

Informan:

Que Cristina Botías Talamantes, licenciada en Biología, ha realizado bajo nuestra dirección y asesoramiento el presente trabajo titulado "PATRÓN EPIDEMIOLÓGICO Y NUEVOS MÉTODOS DE CONTROL DE LAS NOSEMOSIS DE *Apis mellifera* L. EN ESPAÑA", que consideramos reúne las condiciones de calidad científica necesarias para optar al grado de Doctor por la Universidad Complutense.

Que, como resultado de este trabajo se han publicado hasta el momento doce artículos en siete revistas del *Science Citation Index: Applied and Environmental Microbiology, Environmental Microbiology, Environmental Microbiology Reports, Journal of Apicultural Research and Bee World, Journal of Invertebrate Pathology, Parasitology Research* y *Research in Veterinary Science.*

Que, durante la realización de este trabajo, se han presentado 19 comunicaciones a congresos nacionales e internacionales.

De lo que informamos en Madrid, a 24 de Octubre de 2012.

Fdo. Aránzazu Meana Mañes
Profesora Titular de la UCM

Fdo. Mariano Higes Pascual
Asesor Investigación del CAR

Fdo. Raquel Martín Hernández
Investigadora del CAR

“No se debe creer que todos los seres existen para el bienestar del hombre.

Al contrario, todos los demás seres también fueron hechos para su beneficio propio,

y no para otra cosa”

Maimónides (1135-1204)

*A mi familia*

*A Alfonso*

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“a lifetime isn´t enough to love you”…

# ÍNDICE

# INTRODUCCIÓN Y OBJETIVOS

La abeja melífera (*Apis mellifera* Linnaeus 1758) es la especie de abeja más ampliamente distribuida en el mundo, debido en general tanto a su papel crucial en la producción de miel y otros productos de la colmena (jalea real, cera, propóleos, polen, veneno) como a su papel polinizador de cultivos y plantas silvestres a lo largo de todo el globo terráqueo. Su actividad productora y polinizadora convierte a la abeja melífera en uno de los insectos más provechosos para los ecosistemas y para la humanidad.

España cuenta con un gran potencial apícola, ya que se trata de un país en el cual el clima permite que las abejas estén activas la mayor parte del año. Además, cuenta con una gran riqueza florística y con grandes superficies naturales y agrarias donde emplazar las colonias de abejas (García Martínez y col., 2008). En la actualidad nuestro país posee el mayor censo apícola de la Unión Europea, con 2.498.003 colmenas censadas en el año 2011 (19% del censo comunitario; MARM, 2011), presentando asimismo la mayor tasa de profesionalización dentro de la UE y el primer lugar en producción total de miel (15,9% de la producción total de la UE).

Dada la importancia del sector apícola español tanto dentro del sistema agrario nacional como del europeo, se hace imprescindible el mantenimiento de un estado sanitario óptimo en nuestras colonias de abejas que asegure su viabilidad como especie productora y polinizadora, garantizando asimismo su conservación.

Desafortunadamente, la apicultura ha sufrido unas pérdidas muy elevadas de colonias de abejas durante la última década en distintas regiones del mundo, incluido nuestro país (Neumann y Carreck, 2010; COLOSS, 2009a), dándose asimismo un descenso generalizado en todas las actividades apícolas sobre todo en el hemisferio norte (vanEngelsdrop and Meixner, 2009; Potts y col., 2010).

En nuestro país, el equipo de trabajo del Centro Apícola de Marchamalo (CAR) fue alertado por parte de los apicultores sobre un aumento inusual en la mortalidad de colonias a finales de los años 90 y principios del 2000. Más adelante, entre los años 2004 y 2005, este fenómeno comenzó a mostrarse de manera más frecuente y generalizada, y debido a que las colonias que llegaban al colapso mostraban una etiología hasta entonces desconocida, se acordó nombrar esta entidad nosológica como “síndrome de despoblamiento de las colmenas” (Higes y col., 2005). Este

síndrome se relacionó con una serie de manifestaciones, como son los descensos en las producciones de miel y polen, falta de vigor en las abejas, pérdida de población de abejas adultas y en la última fase, un colapso de las colonias cuando ésta no es capaz de mantener la tasa de renovación de la población a un nivel que permita suplir a las abejas enfermas o muertas que dejan de realizar las tareas básicas de la colonia. Sin embargo, la valoración de estas manifestaciones, aunque basada en la observación de un número significativo de colonias, fue realizada de manera subjetiva, por lo que dicho fenómeno precisaba de un estudio más profundo y minucioso.

Por otro lado, durante el mismo período de tiempo en que estas pérdidas generalizadas de colonias de abejas eran reveladas tanto en nuestro país como fuera de nuestras fronteras, en el CAR se observó un aumento en la prevalencia de microsporidios en las muestras clínicas enviadas por los apicultores, sobre todo a partir del año 2003. En ese año, más de un 40% de las muestras recibidas fueron diagnosticadas con nosemosis tras la observación a través de técnicas de microscopía óptica de esporas de microsporidios de morfología compatible con las de *Nosema apis* (Zander, 1909), así como mediante la detección de lesiones macroscópicas e histológicas en las abejas examinadas. En el año 2004 el problema adquirió una mayor magnitud, y sobre 3.000 muestras clínicas de abejas recibidas de diferentes puntos del territorio español se encontró un 90% de las mismas positivas a *Nosema*. Sin embargo, el cuadro clínico presentado por las colonias afectadas de nosemosis no coincidía totalmente con el descrito en la literatura ni el observado en las condiciones experimentales del CAR, ya que las lesiones presentadas en las células del aparato digestivo eran más graves de lo habitual, no se mostraban signos diarreicos en las colonias, y se presentaban brotes de la enfermedad durante el verano, época en la que normalmente desaparecían los casos de nosemosis provocada por *N. apis*.

Por ello, el equipo del CAR se planteó la posibilidad de un cambio en la naturaleza del agente etiológico responsable de la enfermedad. Para confirmar esta hipótesis, se desarrolló una técnica de biología molecular (Reacción en Cadena de la Polimerasa, PCR) que permitió amplificar y secuenciar una parte del genotipo muy específica de los microsporidios con la que poder establecer una diferenciación clara entre especies del género *Nosema* y otros filogenéticamente próximos. De este modo

se comprobó que las secuencias del ADN del gen estudiado presentaban un 100% de homología con la secuencia de la especie *Nosema ceranae* (Fries y col., 1996). Esta detección supuso la primera prueba sobre la capacidad de *N. ceranae* para infectar *A. mellifera* fuera de Asia, y la primera vez que se relacionó con un cuadro clínico concreto (Higes y col., 2006).

Simultáneamente a este hallazgo, *N. ceranae* fue encontrada en colonias de *A. mellifera* en Taiwán (Huang y col., 2007), y más adelante comenzó a confirmarse su presencia en prácticamente todas las regiones del mundo en las que se practica la apicultura (Klee y col., 2007; Martín-Hernández y col., 2007; Chen y col., 2008).

Este microsporidio fue descrito por primera vez como parásito de la abeja asiática *A. cerana* (Fries y col., 1996), y por ello, al no haberse detectado en las poblaciones de *A. mellifera* hasta el año 2005, se sugirió que *N. ceranae* habría invadido este nuevo hospedador entre finales del siglo XX y principios del XIX, propagándose de este modo por las colonias de abejas melíferas europeas tanto en el continente Asiático como en Occidente (Higes y col., 2005; Klee y col., 2007; Martín-Hernández y col., 2007). Sin embargo, dicha hipótesis sobre su origen "oriental" y su condición de patógeno exótico en muchos países occidentales, aunque aceptada por la mayoría de los miembros de la comunidad científica, necesitaba de una confirmación objetiva.

El descubrimiento de un nuevo parásito en la abeja melífera, *N. ceranae*, presumiblemente procedente de latitudes orientales, y la correlación encontrada entre el aumento en el número de muestras de abejas positivas al mismo y el número de colonias de abejas con problemas sanitarios o incluso llegando al colapso, llevó al equipo del CAR a plantearse la hipótesis de una relación causal entre ambos fenómenos. Por ello, se establecieron una serie de ensayos con el fin de estudiar la capacidad patógena de *N. ceranae* tanto en la abeja individual como en la colonia. En el primero de los casos, se demostró la alta capacidad patógena de este microsporidio en abejas infectadas en condiciones de laboratorio, ya que la esperanza de vida de las abejas infectadas se vio significativamente reducida en comparación con aquellas libres de infección (Higes y col., 2007). Este hallazgo fue confirmado por otro equipo de

investigación (Paxton y col., 2007), que además encontró una mayor capacidad patógena de *N. ceranae* con respecto a *N. apis* en abejas infectadas de manera experimental.

A su vez, los estudios basados en el seguimiento de las repercusiones patológicas de la infección natural por *N. ceranae* en las colonias de abejas permitieron confirmar la hipótesis sobre la capacidad de este microsporidio para llevar a las colonias al colapso (Higes y col., 2008a), encontrándose además una serie de signos clínicos y sub-clínicos asociados a dicha patología (*i.e.* largo período de incubación de la enfermedad, descenso en la población de abejas adultas, despoblamiento repentino con presencia de reservas de polen y miel en la colonia). En este ensayo además se descartó la implicación de otros agentes nosógenos como los pesticidas, el ácaro ectoparásito *Varroa destructor*, los virus o el microsporidio *N. apis* en el colapso final de las colonias.

Al mismo tiempo, a través de un estudio epidemiológico realizado por el equipo del CAR en el que se estudiaban los principales agentes nosógenos con una repercusión potencial en la cabaña apícola española, se encontró que la prevalencia de muestras positivas a *Nosema* spp. superaba el 50% tanto en el año 2006 como en el 2007 (Garrido Bailón, 2012). Además, se encontró que las infecciones monoespecíficas producidas por *N. ceranae* suponían el 40%, mientras que *N. apis* se encontraba en un menor nivel (por debajo del 9%). A su vez, las infecciones mixtas de ambos microsporidios fueron también observadas pero siempre por debajo del 7%. Por tanto, este dato revelaba que *N. ceranae* era el agente etiológico más prevalente de las miscrosporidiosis en abejas en nuestro país, y que *N. apis* se mantenía presente en las colonias, aunque en niveles de prevalencia relativamente bajos y conformes a los descritos con anterioridad en nuestro país (CAR, datos no publicados; Gómez-Pajuelo y Fernández-Arroyo, 1979; Orantes-Bermejo y García-Fernández, 1997).

Ante esta situación, en la que se demostraba una marcada diferencia en la prevalencia de ambos microsporidios en nuestro país, así como la alta capacidad patógena de *N. ceranae* para las abejas y las colonias, nuestro equipo de trabajo se planteó los siguientes objetivos:

1. Estudio de los patrones epidemiológicos presentados por *N. ceranae* y *N. apis* en diferentes escalas de análisis, evaluando asimismo los factores que influyen en la epidemiología de estos microsporidios desde el punto de vista del hospedador, del parásito y del medioambiente.

2. Evaluación de diferentes mecanismos de control para las microsporidiosis causadas por *N. ceranae* y/o *N. apis* y seguimiento de la evolución de la parasitación en las colonias infectadas.

# REVISIÓN BIBLIOGRÁFICA

# 1. Los microsporidios

Los microsporidios (Filo Microsporidia) son organismos eucariontes unicelulares, actualmente clasificados dentro del reino de los hongos (Fungi), en el clado Opistokonta (Adl y col., 2005). Son un grupo muy abundante y diverso que cuenta con más de 1200 especies repartidas en unos 150 géneros.

Todos los microsporidios son parásitos intracelulares obligados, los cuales necesitan de un único hospedador para completar su desarrollo (parásitos monoxenos). Su ciclo de vida tiene una fase de merogonia proliferativa, seguida por una fase de esporogonia en la que se producen esporas infectantes que actúan como elemento de resistencia y difusión del organismo. Presentan un rango de hospedadores que incluye a prácticamente todos los filos de invertebrados, especialmente a los insectos (Becnel y Andreadis, 1999; Wittner, 1999), a las cinco clases de vertebrados (Canning y Lom, 1986; Mathis, 2000; Franzen y Muller, 2001; Weiss, 2003) e incluso a ciertos protistas del filo Ciliophora y Apicomplexa (Vivier, 1975).

Aunque son verdaderos eucariotas (con un típico núcleo eucariota, sistema de endomembranas, citoesqueleto), también poseen características moleculares y citológicas más propias de un organismo procariota, como por ejemplo la ausencia de mitocondrias, peroxisomas y las membranas del aparato de Golgi (Mathis, 2000).

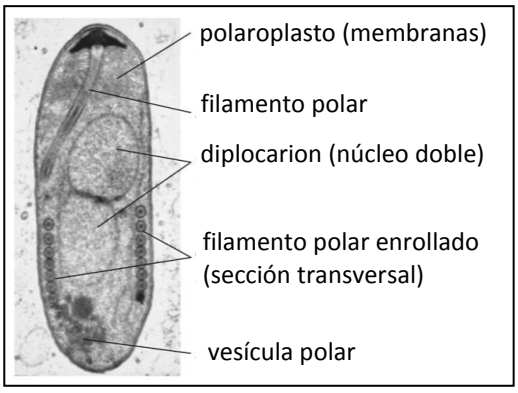


Figura 1. Principales orgánulos de una espora infectiva de microsporidio (Foto de Society for Invertebrate Pathology, extraída de Solter, 2010).

Al igual que otros parásitos intracelulares, los microsporidios están altamente especializados y adaptados a la vida en el medio intracelular, mostrándose muy simplificados tanto en aspectos morfológicos como ultraestructurales, bioquímicos, metabólicos e incluso genómicos en comparación con otros eucariotas (Keeling y Fast, 2002). Además, estos organismos presentan un mecanismo de infección extremadamente sofisticado y único dentro del conjunto de los seres vivos que consiste en un aparato de extrusión de forma tubular (filamento polar) capaz de inyectar el contenido de la espora madura en la célula hospedante.

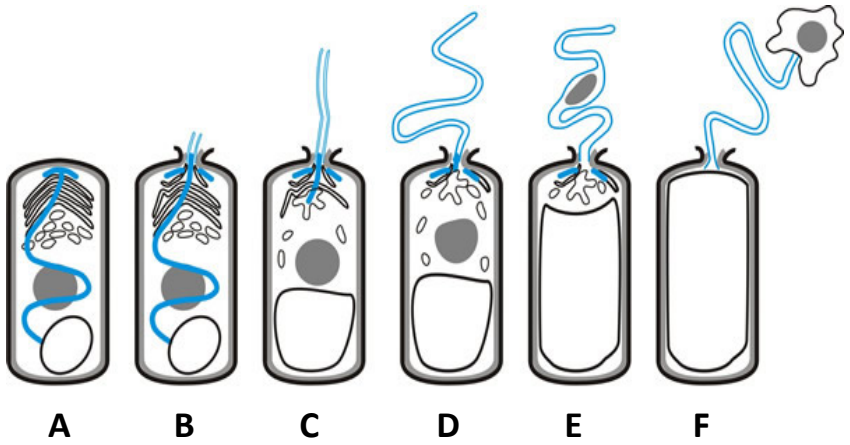


Figura 2. Eversión del filamento polar durante la germinación de la espora de un microsporidio. (A) Espora en estado latente, mostrando el filamento polar (azul), el núcleo (gris), el polaroplasto y la vacuola posterior. (B) El polaroplasto y la vacuola posterior comienzan a hincharse, se rompe el disco de anclaje, y comienza la eversión del filamento polar. (C) El filamento polar continúa su eversión. (D) Una vez el filamento polar ha sufrido una eversión completa, (E) el esporoplasma es forzado a salir a través del mismo. (F) El esporoplasma emerge del filamento polar rodeado por una nueva membrana (basado en ilustraciones de Keeling y Fast, 2002).

Dentro de los microsporidios, el Género *Nosema* comprende más de 150 especies, las cuales suelen infectar hospedadores invertebrados. Pueden aparecer en al menos 12 órdenes de insectos, sobre todo en Lepidoptera e Hymenoptera, y causan ciertas enfermedades bien conocidas como la pebrina en gusanos de seda (provocada por *Nosema bombycis*) y las nosemosis en las abejas melíferas (provocadas por *Nosema* apis y *Nosema ceranae*; Becnel & Andreadis, 1999).

# 2. Nosemosis

Las nosemosis son enfermedades parasitarias caracterizadas por la proliferación de microsporidios del Género *Nosema* en las células epiteliales del ventrículo de las abejas adultas.

Durante casi 100 años se pensó que esta enfermedad estaba causada únicamente por el microsporidio *Nosema apis* en la abeja europea *Apis mellifera* (Zander, 1909; White, 1919), pero más recientemente, otro microsporidio del mismo género, *Nosema ceranae*, fue encontrado como agente infectivo de este hospedador en Europa (Higes et al., 2006) y en Taiwán (Huang et al., 2007). Hasta ese momento, este parásito había sido observado únicamente causando infección en abejas asiáticas (*A. cerana*; Fries y col., 1996), por lo que se cree que éste sería su hospedador natural, y a su vez, la abeja europea sería el hospedador natural de *N. apis*. Aun así, ambos agentes pueden encontrarse en cualquiera de las dos especies de abejas (Chen y col., 2009a), si bien hay que destacar que *N. ceranae* se desarrolla mejor en *A. mellifera* comparada a *N. apis* en *A. cerana* (Fries y Feng, 1995; Fries, 1997).

A pesar de que ambos microsporidios tienen una morfología similar bajo microscopio óptico y una semejanza genética (homología del 92% en la secuencia SSU del ARNr; Fries y col., 1996), las enfermedades causadas por los mismos presentan diferencias en sus patrones epidemiológicos, sintomatología y repercusiones patológicas. Es por ello que serán tratadas como dos patologías independientes (COLOSS, 2009b): nosemosis tipo A producida por *Nosema* apis (Zander, 1909) y nosemosis tipo C producida por *Nosema ceranae* (Fries y col., 1996).

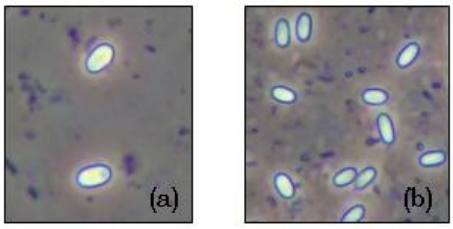


Figura 3. Esporas de *Nosema apis* (a) y *Nosema ceranae* (b) vistas al microscopio óptico con contraste fases a 400 aumentos.

# 2.1. Nosemosis tipo A

La nosemosis tipo A es la enfermedad causada por la proliferación del microsporidio *N. apis* en el interior de las células epiteliales del ventrículo de las abejas adultas de la especie *A. mellifera*.

## 2.1.1. Etiología

*N. apis* es uno de los primeros microsporidios en ser descritos. De hecho, la enfermedad causada por este microsporidio en abejas melíferas se reconoció incluso antes de que el agente etiológico fuera nombrado y descrito, ya que en 1857 Donhöff y Leuckart observaron unos corpúsculos ovales en el ventrículo de abejas muertas, y concluyeron que la causa de la muerte era la exposición a estos agentes patógenos, considerados de naturaleza fúngica por parte de los autores (Donhöff y Leuckart, 1857 revisado por White, 1919). Más adelante, en 1909, Zander describió la presencia de esporas en las paredes del ventrículo de abejas enfermas o muertas, y demostró que éstas eran la causa de una enfermedad que daba lugar a disentería, nombrando al agente etiológico como *N. apis* (Zander, 1909). Los estudios sobre los efectos de la enfermedad en las abejas y en las colonias, así como su transmisión, su posible tratamiento y la resistencia de las esporas en distintas condiciones comenzaron poco después (White, 1914, 1919) y el estudio detallado de los corpúsculos brillantes y ovalados observados en el tracto digestivo de las abejas confirmó el origen fúngico de la enfermedad (Neveu-Lemaire, 1938).

Este microsporidio se transmite por esporas ovales, refringentes, con un tamaño de 5-7 µm de largo, 3-4 µm de ancho. Tiene dos membranas, la exospora proteínica y electrodensa, y la endospora quitinosa y electrolúcida, que la hacen resistente a los factores ambientales adversos. En el extremo anterior del interior de la espora, se encuentran el disco de anclaje donde empieza el tubo polar y el diplocarion rodeado por un filamento polar de 26-32 vueltas (Fries, 1989). En el extremo posterior se aloja una vacuola con material flocular. Todo el conjunto, a su vez, se encuentra rodeado por el esporoplasma.

La infección por *N. apis* se produce cuando las abejas adultas ingieren una espora viable en la alimentación (Bailey, 1981; Webster, 1993), en la trofalaxia (Webster, 1993), mientras realizan tareas de limpieza de la colmena (Fries, 1989; Webster, 1993) o a través del agua (L´Arrivee, 1965). Una vez que las esporas llegan a la luz del ventrículo, éstas germinan, dando comienzo así a la diseminación del parásito (Bailey, 1955). Todavía no se han aclarado las señales que dirigen la germinación, aunque hay una indicación de que el desplazamiento del calcio intracelular y posterior anclaje calcio/calmodulina en la superficie de las esporas podría comenzar una cascada de señales que contribuirían al proceso de hinchamiento y turgencia del polaroplasto (Weidner y Byrd, 1982).

Se desencadena entonces la eversión del filamento polar que adquiere forma de túbulo hueco y penetra en las células intestinales donde inyecta el esporoplasma binucleado (Larsson, 1986). Éste crece en tamaño y madura para convertirse en fase vegetativa o meronte. Aproximadamente 24 horas post-infección se inicia la merogonia. En esta fase es típica la división binaria de los merontes diplocarióticos, aunque también pueden producirse múltiples fisiones para dar lugar a cadenas multinucleadas (Gray y col., 1969). A continuación se forman los esporontes sobre los que se evidencian deposiciones de queratina que darán lugar a la futura exospora. Inmediatamente después, vuelven a dividirse por fisión binaria una sola vez para formar dos esporoblastos, y a partir de ellos se forma una primera generación de esporas binucleadas, que tienen una fina endospora (82-143 nm) y un corto filamento polar. Estas esporas germinan de forma espontánea en el mismo citoplasma de la célula parasitada o en la adyacente (Fries y col., 1992), mecanismo que sirve para propagar la parasitación de una manera rápida a través del epitelio ventricular. A partir del esporoplasma inyectado se obtiene una nueva generación de merontes que, tras múltiples divisiones, da lugar a una segunda secuencia de esporulación. De este modo, los esporontes diplocarióticos resultantes se dividen para dar lugar a dos esporoblastos, que evolucionarán para convertirse en esporas maduras. En este caso, la endospora es mucho más gruesa (278-379 nm) que los de la primera generación y el filamento polar mucho más largo.

Una vez que las células parasitadas están repletas de esporas maduras, éstas pueden romperse liberando las esporas maduras a la luz intestinal, eliminándose posteriormente en las heces de las abejas infectadas (Gilliam y Shimanuki, 1967). Aunque Gray y col. (1969)

indicaron que el ciclo biológico de *N. apis* duraba 7 días, estudios posteriores han confirmado que se completa en 36 horas (Fries y col., 1992).

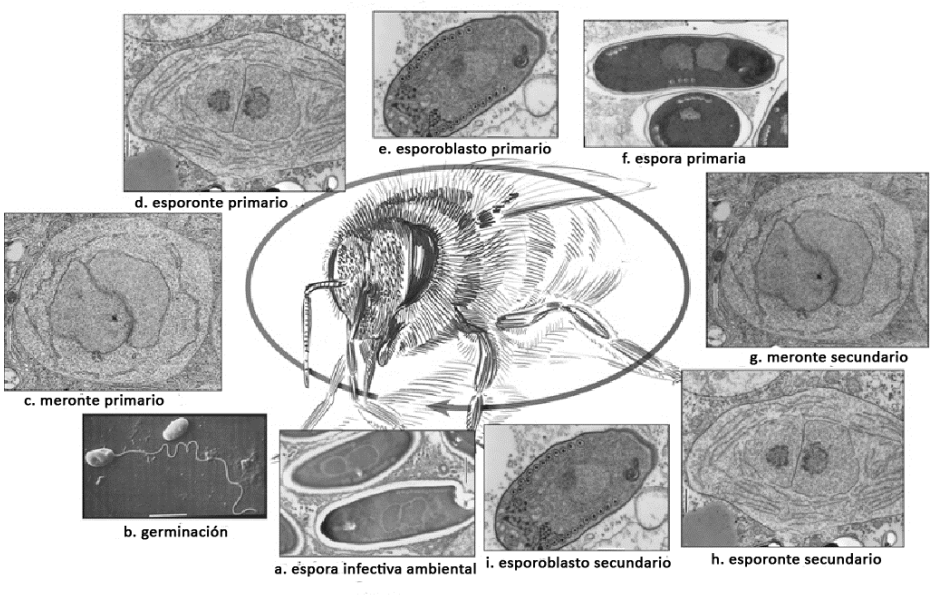


Figura 4. Ciclo de vida de *Nosema* en *A. mellifera* (imagen extraída de Solter, 2010).

## 2.1.2. Patogenia

### a) en la abeja individual

Las abejas parasitadas por *N. apis* no muestran signos clínicos evidentes de la enfermedad e incluso cuando la nosemosis tipo A se presenta de forma aguda, los síntomas son bastante inespecíficos pudiéndose confundir con otras enfermedades de las abejas adultas.

La acción patógena de *N. apis* en las abejas melíferas se centra casi exclusivamente en el epitelio del ventrículo (Bailey, 1981) debido a que el metabolismo de estas células requiere de altos niveles de oxígeno, lípidos y de síntesis proteica para mantenerse, lo cual sería aprovechado por estos microsporidios para su propia proliferación (Liu, 1984). En una abeja en la que la infección se ha desarrollado completamente se pueden encontrar hasta 30-50 millones de esporas en el contenido ventricular (Bailey, 1981). Macroscópicamente el

ventrículo aparece inflamado y presenta un aspecto blanquecino y friable, rompiéndose con facilidad al extraerlo del abdomen (Shimanuki y col., 1992).

Distintos estudios han mostrado la presencia de esporas de *N. apis* en otros tejidos diferentes al ventrículo, como son los ovarios, los túbulos de Malpighi y las glándulas salivares, mandibulares e hipofaríngeas de las abejas (White, 1919, Steche, 1960; Sokolov y Grobov, 1963) sugiriéndose que este microsporidio no presentaría una especificidad para el tipo de tejido a infectar. Más recientemente se detectó y cuantificó la presencia tanto de *N. apis* como de *N. ceranae* en glándulas salivares torácicas, hipofaríngeas y mandibulares, así como en el saco y glándula del veneno de las abejas infectadas utilizando la técnica de PCR cuantitativa a tiempo real (qPCR; Copley y Jabaji, 2011). Sin embargo, ninguno de estos resultados ha sido confirmado por medio de estudios histopatológicos, por lo que hasta el momento únicamente se ha demostrado la presencia de las fases inmaduras de *Nosema*, y por tanto de infección, en las células epiteliales del ventrículo de las abejas adultas. A su vez, no se observó germinación de esporas de este microsporidio en larvas de abejas tras la inoculación de las mismas con esporas de *N. apis* (Hassanein, 1951), aunque posteriormente se encontraron evidencias de infección de las larvas por este microsporidio (Buys, 1972, 1976). Por ello, estos datos deberían ser confirmados con las técnicas de histopatología disponibles en la actualidad para resolver estos resultados contradictorios.

En estudios histológicos se confirmó que las lesiones producidas por la acción citolítica de los merontes se traducen en una disfunción en las células ventriculares del hospedador, extendiéndose la infección desde la parte posterior del ventrículo hacia la anterior (Kellner, 1981; Fries, 1988; De Graaf y col., 1994). Así, las células infectadas por este microsporidio muestran un número inusualmente elevado de vacuolas, de glucógeno residual y de ribosomas agregados, lo cual da evidencias de una extensa lisis celular, aun cuando las membranas celulares aparecen intactas (Liu, 1984). En el curso de la evolución de la enfermedad llegan a afectarse las criptas de regeneración, encargadas de reemplazar las células epiteliales dañadas, apareciendo zonas desprovistas de epitelio a lo largo de todo el tubo digestivo (Muresan y col., 1975; Liu, 1984; De Graaf y col., 1994). Por esta razón las abejas afectadas por *N. apis* acumulan excrementos y presentan el abdomen dilatado y distendido. Como consecuencia final aparece la diarrea (Bailey, 1981), el signo más característico de la nosemosis tipo A. Sin embargo, en algún trabajo se observaron colonias

con altos niveles de infección sin diarrea (Moeller, 1978). En aquellos casos en los que sí aparece, las heces son de color marrón claro casi verdoso y olor fétido, y las abejas las eliminan tanto en el exterior como en el interior de la propia colmena.

Otros síntomas visibles en la abeja son su aspecto brillante (Somerville, 2002), la imposibilidad para volar a consecuencia de la compresión de los sacos aéreos abdominales, y una debilidad generalizada resultado de la malnutrición. Los bajos niveles de proteína, lípidos, glucosa y fructosa con respecto a las abejas sanas (Tomaszweska, 1979) y la anemia ocasionada por la disminución en los elementos formes de la hemolinfa (hemocitos) contribuyen fuertemente a esa debilidad (Gilliam y Shimanuki, 1967) y a una muerte prematura en los casos muy severos de la enfermedad (Morse y Nowogrodzki, 1990). Además, se ha demostrado un menor consumo de oxígeno y agua, y mayor consumo de alimento en las abejas infectadas en comparación con aquellas libres de infección (Moffet y Lawson, 1975). También la infección por *N. apis* disminuye la síntesis de ARN en las células parasitadas, además de inducir cambios en el metabolismo de los ácidos grasos en el ventrículo de las abejas infectadas (Roberts, 1968; Hartwig y Przelecka, 1971; Vandermeer y Gochnauer, 1971).

Asimismo, las abejas parasitadas por *N. apis* sufren un deterioro del metabolismo proteico representado por una menor actividad proteolítica del ventrículo (Malone y Gatehouse, 1998). La disminución de la cantidad de aminoácidos en la hemolinfa causa una reducción de las glándulas hipofaríngeas (Wang y Moeller, 1970, 1971; Liu, 1990) y un descenso de los niveles de proteínas en el cuerpo graso (Lotmar, 1939; Fries y Feng, 1995). A su vez, en las abejas reinas infectadas se detectó una reducción del tamaño de los ovarios, una degeneración de los oocitos (Liu, 1992) y una inhibición de la maduración de los huevos en las ovariolas, presumiblemente debidos a un aporte proteico insuficiente (Hassanein, 1951).

Como consecuencia de la debilidad generalizada que presentan las abejas parasitadas por *N. apis*, aparecen otros síntomas como temblores y parálisis. De hecho, es frecuente encontrar grupos de abejas moribundas o muertas en el suelo, delante de la colmena en colonias con altos niveles de abejas infectadas. De acuerdo a Moeller (1978), las abejas que

se arrastran son sólo características de la enfermedad de los primeros días de la infección de la abeja, en épocas de un gran flujo de miel.

Por otro lado, la accesibilidad de polen fresco no reduce las repercusiones patológicas de *N. apis* en abejas infectadas (Mattila y Otis, 2006; Avilez y Araneda, 2007).

También se ha sugerido la producción por parte de *N. apis* de alguna substancia análoga a la hormona juvenil durante la infección de la célula hospedante, lo cual actuaría interfiriendo en el normal desarrollo de las abejas infectadas y en su paso natural de unas tareas a otras en la colonia (Fisher y Sanborn, 1962).

Todas estas repercusiones patológicas pueden agravarse por la posible acción de diversos virus asociados a *N. apis* como el virus filamentoso o el de las realeras negras (Bailey, 1982), o en presencia de insecticidas como la imidacloprida (neonicotinoide; Alaux y col., 2010).

### b) en la colonia

Al igual que ocurre en la abeja individual, en las colonias afectadas de nosemosis tipo A no se observan signos evidentes de enfermedad.

La forma latente de la nosemosis tipo A es más habitual durante el invierno y a pesar de que no presenta ningún síntoma evidente, la fisiología de las abejas está alterada. En las colonias afectadas se observa una disminución en el número de abejas de la colonia y menor producción de cría (Yücel y Dogaroglu, 2005), un mayor consumo de alimento y una falta de dinamismo en primavera. Asimismo, estas colonias tienen un menor rendimiento en la producción de miel (Farrar, 1947; Fries y col., 1984) y experimentan una disminución significativa en la capacidad de recolección de polen (Anderson y Giacon, 1991).

En colonias infectadas con *N. apis* se ha descrito un envejecimiento prematuro de las abejas, lo que lleva a una reducción de la longevidad de las mismas (White, 1919; Furgala y Mussen, 1990). Estos efectos además están acompañados por cambios en la división de tareas y precocidad en el pecoreo (Wang y Moller, 1970) que afecta al comportamiento de las abejas. Woyciechowski y Kozlowski (1998) también demostraron un incremento del pecoreo de obreras afectadas con *N. apis* en condiciones meteorológicas adversas.

Asimismo, en aquellas colonias altamente infectadas, la atrofia en las glándulas hipofaríngeas de las abejas infectadas puede traducirse en un descenso en su capacidad de secreción de jalea real o en el contenido proteico de la misma, lo que provocaría un déficit alimentario en aquellas larvas que se encuentran en las primeras etapas del desarrollo y son alimentadas con esta sustancia. Como consecuencia, la cantidad de huevos que dan lugar a abejas maduras puede llegar a disminuir hasta en un 15% (Hassanein, 1951; Wang y Moeller, 1969), lo que en última instancia puede contribuir al descenso en el tamaño de la población.

En las colonias altamente infectadas también se ha descrito una mayor tendencia a reemplazar la reina de manera prematura (Moeller, 1962; Loskotova y col., 1980), y se ha demostrado que las reinas infectadas disminuyen su capacidad de puesta, lo que se traduce en hasta un 12% menos de producción de cría. Sin embargo, las probabilidades de infección de la reina son moderadas en las colonias infectadas (Furgala, 1962) debido a que las abejas que la rodean no suelen estar parasitadas (Wang y Moeller, 1970), y únicamente se incrementan en condiciones de confinamiento de la reina durante largos periodos de tiempo con abejas infectadas (Foote, 1971; Lehnert y col., 1973). Por otro lado, la probabilidad de infección de aquellas obreras que se encuentran dentro de una colonia en la cual la reina está infectada se incrementa de manera significativa (Czekonska, 2000).

### 2.1.3. Epidemiología

En la actualidad, la nosemosis tipo A está expandida mundialmente (Furgala y Mussen, 1990; Matheson, 1996) siendo la enfermedad más distribuida de las abejas adultas (Nixon, 1982), aunque su prevalencia es menor en áreas tropicales y subtropicales (Wilson y Nunamaker, 1983). Su rango de hospedadores comprende a dos especies, *A. mellifera* y *A. cerana*, siendo más prevalente en la primera de ellas (Chen y col., 2009a).

Cuando la enfermedad se presenta de forma aguda, puede producir un impacto desfavorable en la producción de miel, en la polinización y en la cría de abejas lo que conlleva grandes pérdidas económicas (Hornitzky, 1985; Anderson y Giacon, 1992).

La nosemosis tipo A afecta tanto a la reina como a las obreras y a los zánganos (White, 1919; Fries, 1989, 1993; Bailey y Ball, 1991), influyendo de forma notable la edad. De

hecho, se ha demostrado que las abejas viejas son más propensas a sufrir la enfermedad, mientras que las abejas menores de 15 días están menos expuestas a la infección debido a su etología en esa etapa , tienen menos probabilidades de ser infectadas debido a contraer la infección al son menos receptivas (Wang y Moeller, 1970).

Los efectos de *N. apis* en reinas son particularmente importantes para la práctica apícola. La diminución en el desarrollo de los ovarios dirige a una infertilidad (Fyg, 1964; Liu, 1992) y consecuentemente a una sustitución de la reina (Farrar, 1947; Furgala, 1962; Czekonska, 2000), lo cual puede acarrear importantes pérdidas económicas para el apicultor.

En la abeja asiática *A. cerana*, *Nosema* se desarrollan menos que en las abejas europeas, siendo la parasitación autolimitante en este hospedador lo que determina una menor patogenicidad. En las abejas melíferas europeas, la parasitación por *N. apis* no es autolimitante, progresando con rapidez en las células epiteliales del ventrículo, fundamentalmente en las abejas más adultas (pecoreadoras). En las últimas fases de la enfermedad, en la colonia también se afecta la reina, lo que compromete la viabilidad de la colonia de abejas.

La dosis infectiva de *N. apis* está cerca de 100 esporas por abeja (Fries, 1989) y la esperanza de vida de las obreras afectadas se reduce entre un 22 y un 44% respecto de las obreras sanas (Kang y col., 1976) lo cual, durante la primavera y el verano, podría causar la escasez de las abejas de mayor edad. De este modo, la colonia no puede construir tan rápida y vigorosamente como lo haría en condiciones normales (Delaplane, 1998).

Las abejas de diferentes estaciones poseen características fisiológicas distintas que pueden influir en su receptividad al parásito. Las abejas de primavera y otoño son las más parasitadas por *N. apis* (Bailey, 1955). En la parasitación por este microsporidio, hay una clara influencia de las condiciones climáticas (Webster, 1993). Las bajas temperaturas del invierno hacen que el parásito se multiplique más lentamente, resultando difícil encontrar esporas en el ventrículo de las abejas, a no ser que las abejas estén fuertemente infectadas. Tan pronto como se eleva la temperatura en primavera y comienza la cría de larvas y la ingestión de proteínas, el parásito se multiplica masivamente (Pickard y El-Shemy, 1989). Es en este momento de intercambio de abejas de invierno a abejas de verano cuando suele desarrollarse clínicamente la nosemosis tipo A. Si las abejas no pueden salir de la colmena

por las bajas temperaturas se facilita el contacto entre las heces contaminadas y el alimento, aumentando la transmisión de la enfermedad y provocando la aparición de casos clínicos. En caso de que la población infectada sobreviviese, según avanza la primavera, las abejas más afectadas que salen a volar raramente regresan. Disminuye así la contaminación en el interior, lo que unido a la rápida sustitución de abejas viejas afectadas por abejas nuevas y la corta vida activa de las abejas de verano, hace que el parásito se multiplique menos y se reduzca la carga parasitaria. También la abundancia de precipitaciones parece ser un factor favorecedor para el mayor desarrollo de *N. apis* en las colonias de abejas (Fries, 1988; Aydin y col., 2006). En veranos muy lluviosos pueden repetirse los factores del inicio de la primavera y aparecer prevalencias elevadas en el otoño. En estos casos, las abejas van muriendo durante la pausa invernal y la colmena es incapaz de sobrevivir el invierno (Bailey, 1955). No obstante, este hecho resulta excepcional en la parasitación por *N. apis*.

Las esporas de *N. apis*, responsables de la transmisión de la enfermedad, pueden permanecer viables varios meses en la miel (White, 1919; Moeller, 1978), aunque la infectividad no se mantiene por un largo plazo (Malone y col., 2001), y también en los cadáveres de las abejas infectadas (Steche, 1985) e incluso más de un año en las heces de abejas (Bailey, 1962). Asimismo, es probable que la contaminación fecal de la cera, en especial en los panales utilizados para la cría, o de otras zonas de la colmena, proporcione suficiente cantidad de esporas para que la enfermedad se transmita a la nueva generación de abejas. La importancia relativa de estos reservorios no se ha estudiado en profundidad, pero parece que la temperatura puede afectar a la pérdida de viabilidad de las esporas, independientemente del medio en el que se encuentre (Morgenthaler, 1939). Otro reservorio de esporas viables pueden ser las zonas húmedas donde las abejas recogen agua, ya que las esporas de otros microsporidios permanecen viables largos periodos de tiempo en este medio (Koudela y col., 1999; Fournier y col., 2000; Fayer, 2004).

## 2.1.4. Diagnóstico

La ausencia de signos patognomónicos en la nosemosis tipo A y la obligatoriedad de comunicar esta enfermedad a la UE (lista C del anexo I del RD 617/2007) hace que sea necesaria la confirmación laboratorial del diagnóstico. Para ello la OIE (2008) propone algunos métodos:

### a) Examen macroscópico

Se realiza una disección de las abejas parasitadas para hacer un examen de los ventrículos. Éstos aparecen blanquecinos y friables en grado variable, aunque esta apariencia puede ser debida a otros problemas intestinales (alimentación con jarabes con gran cantidad de levaduras en multiplicación o infección por virus epiteliotropos como el virus Kashmir o el virus de las realeras negras).

### b) Examen microscópico

Se puede realizar un examen microscópico cuantitativo o no. En ambos casos, se recogen abejas de más de 10-15 días, a ser posible pecoreadoras, para evitar resultados falsos negativos, ya que en abejas jóvenes el microsporidio puede no haber producido un número suficiente de esporas maduras que sean detectables.

Para el método no cuantitativo, se aconseja recoger al menos 60 abejas con el fin de detectar el 5% de las abejas enfermas con un 95% de confianza (Fries, 1989), ya que muestras de menor tamaño pueden dar como resultado final del diagnóstico falsos negativos. Se separan los abdómenes de las abejas, se maceran en agua destilada estéril, se centrífuga y tres gotas del sedimento resuspendido en agua destilada estéril, se coloca en un portaobjetos bajo un cubreobjetos para hacer una observación en fresco a 400 aumentos, en un microcopio de contraste de fases (Cantwel, 1970). En estas condiciones las esporas de *Nosema* aparecen refringentes, con un contorno bien definido y sin diferenciarse su contenido interno, lo que permite no confundirlas con levaduras, que no son refringentes y se suele observar un contenido granuloso en su interior. Las esporas de *N. apis* son ovales con polos más redondeados y más grandes que las de *N. ceranae*, que aparecen más alargadas y con los polos menos redondeados. Además es importante distinguir entre una

infección por *N. apis* y una infección causada por *Malpighamoeba mellificae* (Webster, 1993). Los quistes de *M. mellificae* son esféricos y de aproximadamente 6-7 um de diámetro.

En caso de ser necesaria la aplicación de tinciones, está recomendada la tinción con Giemsa. Las esporas de *N. apis* tienen una apariencia distintiva con una pared gruesa no teñida y un interior azul, sin núcleos visibles.

El método cuantitativo es similar al anterior pero en este caso sólo se utilizan 10 abejas. Se maceran en agua destilada estéril y posteriormente se centrifuga, el sedimento se resuspende en agua destilada estéril. Una vez homogeneizado el macerado, se llena una cámara de recuento (hemocitómetro) y se cuenta el número de esporas, que es orientativo del grado de parasitación de la muestra y no está directamente relacionado con el grado de parasitación real de la colmena.

### c) Identificación molecular

La OIE (2008) recomienda una técnica de PCR múltiple de la región *small-subunit* del gen 16S del ARN ribosómico (SSU-16S ARNr) desarrollada en nuestro centro (Martín-Hernández y col., 2007), con la que se pueden identificar simultáneamente ambos tipos de microsporidios (*N. apis* y *N. ceranae*).

- Preparación de la muestra:

Los abdómenes de 20 abejas adultas de cada muestra son macerados en 10 ml de agua destilada y la suspensión se filtra y centrifuga a 800 g durante 6 minutos. Para la extracción del ADN se induce la germinación con 200 µl de buffer de germinación (0.5 M de cloruro de sodio, 0.5 M de carbonato de sodio, y pH de 6.0 con ácido ortofosfórico), y la mezcla se incuba a 37°C durante 15 minutos. La extracción de ADN se puede llevar a cabo por medio de un kit comercial, como por ejemplo “High Pure PCR Template Preparation Kit” (Nº catálogo 1796828 Roche Diagnostic).

- Reacción en Cadena de la Polimerasa (PCR) Múltiple:

Mediante esta técnica, ambos microsporidios (*N. apis* y *N. ceranae*) pueden ser identificados y distinguidos el uno del otro en una única PCR gracias al uso de cebadores específicos que no presentan interferencias entre ellos. Cada reacción de PCR se realiza en

un volumen de 50 µl, conteniendo 5 µl de ADN de la muestra, 25 µl de polimerasa (High Fidelity PCR Master Mixture, Nº catálogo 12140314001, Roche Diagnostic), 0.4 µM de cada cebador, 0.4 µM de cada desoxirribonucleósido trifosfato (dNTP), 3 µM de Cl2Mg, 0,2 mg/ml de albúmina de suero bovino (BSA), 0.1% de Tritón X-100, y 5 µl de ADN de *N. ceranae* o *N. apis* para los controles positivos.

Los parámetros para la amplificación son: un paso inicial de activación de la PCR de 2 minutos a 94°C, seguido de 10 ciclos de 15 segundos a 94°C, 30 segundos a 61.8°C, y 45 segundos a 72°C, y 20 ciclos de 15 segundos a 94°C, 30 segundos a 61.8°C, y 50 segundos a 72°C más un ciclo de elongación de 5 segundos añadido a cada ciclo sucesivo, y por último un paso final de extensión a 72°C durante 7 minutos. Los controles positivos (con ADN de *N. apis* y *N. ceranae*) y negativos (procedentes de la extracción de ADN) se incluyen en cada una de las PCRs llevadas a cabo.

Los pesos moleculares de los productos de PCR se determinan por electroforesis en un gel de agarosa 2% TAE (Tris-acetato-ácido etilendiamino tetraacético) en un buffer TAE, marcado con bromuro de etidio, y visualizado utilizando luz ultravioleta (UV).

Los cebadores utilizados para detectar *N. apis* y *N. ceranae* en PCR múltiple se muestran en la Tabla 1:

| CEBADOR | SECUENCIA[a] | TAMAÑO PRODUCTO PCR (pb) | ESPECIE |
|---|---|---|---|
| 218MITOC-FOR | 5´-CGGCGACGATGTGATATGAAAATATTAA-3´ | 218-219[b] | *N. ceranae* |
| 218MITOC-REV | 5´-CCCGGTCATTCTCAAACAAAAAACCG-3´ | | |
| 321APIS-FOR | 5´-GGGGGCATGTCTTTGACGTACTATGTA-3´ | 321 | *N. apis* |
| 321APIS-REV | 5-GGGGGGCGTTTAAAATGTGAAACAACTATG-3´ | | |

a Las colas CG añadidas en los cebadores están subrayadas

b Hay 1 pb de diferencia entre los amplicones obtenidos de *N. ceranae*, dependiendo de las diferentes secuencias dispobibles en GenBank (http://www.ncbi.nlm.nih.gov).

En la actualidad, este método (Martín-Hernández y col., 2007) ha sido optimizado con la inclusión de un control interno de PCR (IPC), que consiste en un cebador específico para el gen de la subunidad I de la Citocromo Oxidasa (COI) de *A. mellifera* en la misma PCR múltiple. Este IPC se ha incluido con el fin de poder identificar aquellos resultados negativos de las reacciones que pudiesen ser debidos a algún tipo de fallo químico o instrumental (falsos negativos), ya que ADN de *A. mellifera* siempre es co-extraído de las muestras (infectadas o no). La metodología de esta técnica se explica con detalle en el artículo 1 de esta memoria (Martín-Hernández y col., 2012).

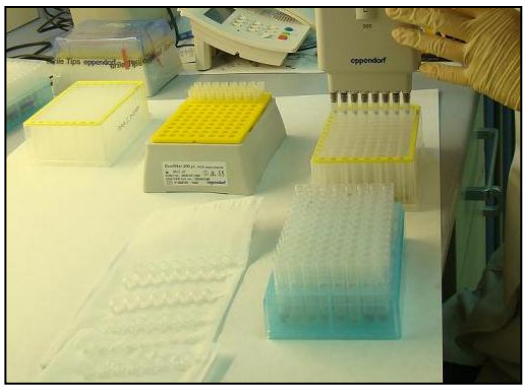


Figura 5. Método de extracción de ADN mediante el cual varias muestras de abejas son procesadas de manera simultánea.

No obstante, para la detección de *Nosema* spp. se ha descrito un amplio número de métodos moleculares utilizando cebadores específicos (Cox-Foster y col., 2007; Chen y col., 2008; Suwannapong y col., 2011). Además técnicas de PCR-RFLP (Klee y col., 2007; Tapaszti y col., 2009) y PCR a tiempo real (Chen y col., 2009a; VanEngelsdorp y col., 2009; Bourgeois y col., 2010; Burgher-MacLellan y col., 2010; Traver y Fell, 2011).

## 2.1.5. Control

El producto activo más utilizado para el control de las nosemosis es la fumagilina (biciclohexilamonio fumagilina; Katznelson and Jamieson, 1952; Cantwell y Shimanuki, 1970; Fries, 1997; Nozal y col., 2008), la cual se obtiene del hongo *Aspergillus fumigatus* (Bailey, 1955; Webster, 1994; Didier, 1997) y es una de las pocas moléculas activas frente a los microsporidios (McCowen et al. 1951). Se trata del único medicamento registrado en

Estados Unidos y en Canadá para tratar las infecciones por *Nosema* spp. en las colonias de abejas melíferas. En la Unión Europea (UE), este antibiótico ha sido utilizado bajo supervisión veterinaria durante varios años, y en nuestro país fue utilizado por medio de una autorización temporal excepcional entre los años 2005 y 2008, pero en la actualidad su utilización no está permitida en las colonias de abejas de la UE por no estar establecidos los límites máximos de residuos para la sustancia activa.

Este antibiótico actúa únicamente sobre la forma vegetativa de *Nosema* spp., en el interior de las células del epitelio ventricular, favoreciendo la reepitelización de las zonas afectadas (Mladjan y col., 2000a, b). El tratamiento con fumagilina inhibe la replicación del ADN en las células del parásito, lo cual, a su vez, permite restablecer las tasas de síntesis de ARN en las células epiteliales del ventrículo de las abejas (Hartwig y Prezelecka, 1971). Asimismo, esta molécula es capaz de inhibir la incorporación de lípidos procedentes de las células hospedantes por parte de *Nosema* spp., alterando de este modo la formación de las membranas en las formas inmaduras de estos microspordios (Liu, 1973) y limitando de este modo la proliferación del parásito en el ventrículo de las abejas.

Los meses otoñales son el mejor momento para tratar las colonias (Bailey, 1955), debido a que la cantidad de abejas adultas es menor en esta época y el tratamiento permite que éstas pasen el invierno libres de infección. Es interesante destacar la alta inestabilidad de la fumagilina bajo radiación, lo que implica una rápida degradación de la misma, teniendo la temperatura un efecto menor aunque también notable (Nozal y col., 2008). Por ello, el jarabe medicado no debe exponerse a altas temperaturas ni a la acción directa de la luz ultravioleta.

Por otro lado, ciertos estudios han mostrado un efecto genotóxico y mutagénico de la fumagilina en células de mamíferos en condiciones experimentales (Stanimirovic col., 2007; COM, 2011), por lo que el establecimiento de los límites máximos de residuos en miel de esta molécula serían necesarios en aquellas regiones en las que su aplicación en las colonias de abejas está permitida.

Otros productos que se han presentado como posibles agentes terapéuticos para el control de la nosemosis tipo A son el timol (2- iso propil-5-metilfenol; Rice, 2001; Yücel y Dogaroglu, 2005), consituyente del aceite esencial de tomillo, o el ácido fórmico

(Underwood y Currie, 2009), mientras la aplicación de ácido benzoico en las colonias (Forsgren y Fries, 2005) no ha mostrado actividad frente a esta enfermedad.

En cualquier caso, antes de recurrir a un tratamiento veterinario, lo mejor es prevenir la aparición de la nosemosis tipo A. Para ello, se hace necesario eliminar aquellos factores que favorecen la aparición de la enfermedad, manteniendo una buena higiene en el colmenar, vigilando en particular las reservas alimenticias y controlando la fecundidad de la reina para asegurar una renovación efectiva de la población de abejas (Moeller, 1972; Loskotova y col., 1980). Además, el mantenimiento de las colonias a temperaturas superiores a los 37° C durante largos periodos de tiempo permiten controlar la enfermedad, ya que a estas temperaturas la proliferación de *N. apis* se ve inhibida (Burnside y Revell, 1948).

Asimismo, el apicultor debe favorecer los vuelos invernales en las colonias infectadas con el fin de promover la defecación de las abejas enfermas fuera de la colmena, disminuyendo de este modo la contaminación de los cuadros y panales (Moeller, 1972), aunque esta operación sólo debe ser practicada cuando las condiciones meteorológicas lo permitan. Las manipulaciones excesivas a principios de primavera también deben ser evitadas cuando las condiciones meteorológicas no son plenamente satisfactorias, siendo ésta la época en la que se deben eliminar las colonias muy débiles. Además es necesario realizar una renovación de los cuadros y una desinfección del material de manera periódica, ya que la reutilización sistemática de material apícola incrementa las posibilidades de desarrollar nosemosis tipo A en las colonias (Fries, 1988). Esta desinfección debe realizarse con ácido acético y derivados (Bailey, 1957; De Ruiter y Van Der Steen, 1989), pudiéndose efectuar también mediante la fumigación del material con óxido de etileno (ETO).

Es conveniente realizar una monitorización sistemática de los colmenares ubicados en zonas con gran prevalencia de nosemosis para poder adoptar las medidas sanitarias adecuadas.

# 2.2. Nosemosis tipo C

La nosemosis tipo C es una enfermedad de las abejas melíferas causada por *N. ceranae* (COLOSS 2009b, Higes y col., 2010). Este microsporidio fue descrito por primera vez en el año 1996 como parásito de la abeja asiática *A. cerana* (Fries y col., 1996) y más adelante, en el año 2005, infectando a la abeja melífera europea *A. mellifera* (Higes y col., 2005, 2006; Huang y col., 2007).

Gran parte de los trabajos sobre *N. ceranae* en Europa se han visto estimulados por un grupo de trabajo europeo reunido inicialmente en Wageningen (2007) denominado "Prevención de las pérdidas de abejas en Europa". Está constituido como una EU COST Action (FA0803) con más de 150 miembros de 40 países (incluidos también países de fuera de la EU) altamente organizado y conocido como COLOSS Group (Neumann y Carreck, 2010).

## 2.2.1. Etiología

Al igual que en el caso de la nosemosis tipo A, los elementos de difusión de *N. ceranae* son las esporas. Morfológicamente son similares a las de *N. apis*, aunque de menor tamaño (3,3-5,5 x 2,3-3 µm) (Fries y col., 1996; Chen y col., 2009b). También son binucleadas (Fries y col., 1996, 2006; Higes y col., 2007), pero el filamento polar es considerablemente más corto (18-23 vueltas) (Fries y col., 1996; Higes y col., 2007, 2008b; Chen y col., 2009b). Todo ello está rodeado por una exospora y endospora con una separación entre ambas de 14-17 nm (Fries y col., 1996). *N. ceranae* mantiene el contacto directo entre el plasmalema y el citoplasma de las células hospedantes faltando algún tipo de envoltura interfacial como vesículas parasitóforas o esporóforas (Higes y col., 2010). El mecanismo de infección de *N. ceranae* en las células epiteliales del ventrículo de *A. mellifera* y las fases inmaduras presentadas por *N. ceranae* son equivalentes a las descritas para *N. apis* en el apartado 2.1 de esta memoria.

Las esporas de *N. ceranae* son más resistentes a la desecación y tienen una mayor termotolerancia a 60 °C durante seis horas (Fenoy y col., 2009) comparado con las esporas de *N. apis* que mueren tras 15 min a 60 °C (Cantwell y Shimanuki, 1970). Por el contario,

después de la congelación, las esporas de *N. apis* muestran un índice de infectividad mayor que las de *N. ceranae* (Forsgren y Fries, 2010) aunque si éstas se mantienen en un medio de conservación adecuado (RPMI 10% + DMSO con o sin suero fetal bovino), la pérdida de viabilidad no es tan dramática y pueden mantenerse viables más del 80% de las esporas congeladas durante tres semanas (Fenoy y col., 2009).

En cuanto a su material genético, el ADN ribosomal está organizado en unidades repetidas en tándem, al igual que ocurre en *N. apis* (Gatehouse y Malone, 1998), cada una de ellas formada por una subunidad ribosomal pequeña (SSU), un espaciador transcrito interno (ITS), una subunidad ribosomal grande (LSU) y un espaciador intergénico (IGS). El gen del ARN 5S se localiza al final de la LSU, y está seguido por el IGS (Huang y col., 2008).

Aunque la caracterización de las especies o las distintas cepas tras el análisis de las secuencias repetidas asume una identidad de todas las repeticiones, éstas no son idénticas en el caso de *N. ceranae* ni siquiera dentro de la misma célula (Sagastume y col., 2010). Esta heterogeneidad de secuencias también ha sido observada en *N. bombi* (Tay y col., 2005; O´Mahony y col., 2007) y podría ocurrir en *N. apis* (Gatehouse y Malone, 1998), lo cual sugiere la presencia de recombinación, y por tanto, de la existencia de alguna etapa diploide en el ciclo celular de los microsporidios del género *Nosema* (Sagastume y col., 2010), lo que supondría una ventaja evolutiva para los mismos.

La amplia diversidad observada en las secuencias de rADN en los extractos de *N. ceranae* complica la determinación taxonómica y el establecimiento de relaciones filogenéticas entre especies, por lo que se hace necesaria la búsqueda de otros marcadores genéticos, que idealmente deberían ser de copia única (Fries, 2010; Sagastume y col., 2010; Hatjina y col., 2011).

## 2.2.2. Patogenia

### a) en la abeja individual

Debido al hallazgo relativamente reciente de *N. ceranae* como parásito de *A. mellifera*, todavía se desconocen muchos aspectos sobre esta patología e incluso muchos de

los que se saben, se han asumido similares a *N. apis*. Por lo tanto, aún se necesitan muchos estudios que permitan esclarecer los rasgos propios de esta patología.

El desarrollo de ambas especies dentro de las células del ventrículo parece ser similar (Fries y col., 1996: Higes y col., 2007; Chen y col., 2009b). En menos de tres días después de la infección, se pueden observar tanto esporas maduras como esporas vacías dentro de las células infectadas (Higes y col., 2007). Esto indica que la germinación intracelular de esporas con *N. ceranae* tiene lugar en *A. mellifera* por una transmisión horizontal entre células epiteliales, de manera similar a *N. apis*. Hasta ahora, en *A. cerana* no se había detectado la presencia de esporas de *N. ceranae* de germinación intracelular (Fries y col., 1996), lo que implicaría una menor capacidad de diseminación del parásito, apareciendo las células parasitadas aisladas y rodeadas de célula sanas libres del parásito. Sin embargo, en la abeja europea parece que el parásito se multiplica igual que *N. apis*, aunque se ha descrito una invasión profunda de los tejidos que afecta incluso a las células de regeneración, lo que tiene importantes repercusiones sobre la capacidad de recuperación del epitelio.

La primera infección experimental de *A. mellifera* por *N. ceranae* (Higes y col., 2007) mostró que este parásito era altamente patógeno para su nuevo hospedador y que además inducía una mortalidad mayor que *N. apis* (Paxton y col., 2007). Las lesiones producidas por *N. ceranae* en el tejido epitelial del ventrículo de su hospedador (órgano diana de la infección) son similares a las producidas por *N. apis*, aunque muestran ciertas diferencias, como por ejemplo una presencia inusual de lisosomas (muchos de ellos secundarios) en el citoplasma y rotura de las membranas celulares, fenómenos no observados en la infección por *N. apis* (Liu, 1984). También se observa una condensación y reducción del tamaño de los núcleos celulares, y las células epiteliales más parasitadas muestran los núcleos desplazados apicalmente con algunas fases inmaduras y maduras de *N. ceranae* en las invaginaciones de la membrana nuclear, tal y como se había descrito anteriormente para *N. apis* (De Graaf y col., 1994). Estas lesiones se aprecian tanto en abejas infectadas y mantenidas en condiciones experimentales (Higes et al., 2007) como en abejas infectadas de manera natural en condiciones de campo (Higes y col. 2008, 2009; García-Palencia y col., 2010).

Por otra parte, otros estudios sugieren que *N. ceranae* tendría capacidad de infectar otros tejidos diferentes al ventrículo, como son los túbulos de Malpighi, las glándulas

hipofaríngeas, las glándulas salivares (Chen y col., 2009b; Gisder y col., 2010), y el saco y la glándula del veneno (Copley y Jabaji, 2012). Estos resultados han sido obtenidos tras realizar un análisis de PCR de los citados tejidos, sin haberse corroborado hasta el momento esta hipótesis con estudios histopatológicos que demuestren la posible infección de dichos tejidos por parte de *N. ceranae*.

Además de las alteraciones anatomopatológicas, también han sido descritas alteraciones en la capacidad de vuelo de las abejas infectadas (Krajl y Fuchs, 2010), disminuyendo la tasa de abejas que son capaces de volver a la colmena tras realizar sus tareas de pecorea y muriendo por tanto lejos de los colmenares, tal y como se ha demostrado en condiciones de campo (Higes y col., 2008a). Este efecto negativo sobre la capacidad de vuelo podría estar relacionada con la reducción en los niveles de carbohidratos en la hemolinfa de las abejas infectadas por *N. ceranae* (Mayack y Naug, 2010; Aliferis y col., 2012), lo cual llevaría a un descenso en la disponibilidad de fuentes de energía para estos insectos. Asimismo, han sido descritas otras alteraciones en el comportamiento en relación con el estrés energético inducido por la infección de este microsporidio (Mayack y Naug, 2009; Naug y Gibbs, 2009; Martín-Hernández y col., 2011), apreciándose que las abejas infectadas tienen un mayor grado de demanda y apetencia por el alimento y como consecuencia, la tasa de ingestión de carbohidratos se incrementa y la de trofalaxia se reduce en comparación con las abejas libres de infección. Asimismo, y también como consecuencia del estrés energético inducido por la infección, se encontró que las abejas infectadas por este microsporidio presentan un descenso en la capacidad termorreguladora y por ello, una preferencia significativa por situarse en las áreas más cálidas de la colonia (Campbell y col., 2010).

Además de los efectos anteriormente descritos, diversos estudios se han centrado en las repercusiones de la infección del microsporidio *N. ceranae* en la fisiología de la abeja. En este sentido, se ha detectado un aumento significativo en la producción de la feromona primaria etil o leato (OE) en las abejas infectadas por *Nosema* (Dussaubat y col., 2010). Esta feromona participa en la regulación de la maduración de las abejas obreras y por tanto en la transición de abeja de interior a pecoreadora (Leoncini y col., 2004), incrementándose en condiciones normales los niveles de EO en las abejas pecoreadoras con respecto a las nodrizas y otras abejas de interior. En el caso de las abejas infectadas, este aumento

irregular en los niveles de EO podría llevar a la maduración precoz de las mismas. Además, debido a que se trata de una feromona primaria, y por ello esencial para la regulación del comportamiento social y la homeostasis de la colmena, una modificación en sus niveles podría alterar la organización de la colmena y poner en peligro su desarrollo y supervivencia. También se ha descrito un aumento en el estrés oxidativo en el ventrículo de las abejas infectadas, así como una inhibición en la expresión de genes implicados en la homeostasis y renovación de los tejidos ventriculares (Dussaubat y col., 2012).

La infección en las abejas reinas produce lesiones histológicas similares a las descritas para las abejas obreras (Higes y col., 2009a), y fisiológicamente se observa un incremento en los niveles de vitelogenina (indicador de fertilidad y longevidad), en la capacidad total antioxidante y en los niveles de feromonas mandibulares (Alaux y col., 2011). Estos cambios fisiológicos podrían alterar el estado sanitario de las abejas reinas, y por tanto, de la colonia.

Otro efecto patógeno provocado por *N. ceranae* es la supresión parcial de la respuesta inmune humoral y celular de las abejas infectadas (Antúnez y col., 2009; Chaimanee y col., 2012), dándose una reducción en la expresión de genes codificantes para péptidos antimicrobianos y otras enzimas relacionadas con la inmunidad. Como consecuencia, otros patógenos como los virus ARN de las abejas aumentarían su capacidad de replicación. Sin embargo, mediante un estudio preliminar se ha sugerido la existencia de una correlación negativa entre *N. ceranae* y el virus de las alas deformadas (Costa y col., 2011), achacando este efecto antagónico a la competición entre ambos patógenos por los mismos recursos, las células epiteliales del ventrículo de las abejas. De este modo, las lesiones y degeneración en las células ventriculares causadas por la infección de *N. ceranae* (Higes y col., 2007) podrían interferir en el curso de la patogénesis del DWV de manera crítica (Boncristiani y col., 2009). Por otro lado, otros autores han señalado la acción sinérgica entre virus ADN (iridovirus) y *N. ceranae* para llevar a las colonias de abejas al colapso (Bromenshenk y col., 2010).

En cuanto al impacto de la infección por parte de este microsporidio sobre la esperanza de vida de las abejas obreras, distintos estudios han demostrado su alto grado de patogenicidad al comprobar la mayor tasa de mortalidad presentada por las abejas infectadas en comparación con aquellas libres de infección (Higes y col., 2007; Paxton y col.,

2007; Martín-Hernández y col. 2009, 2011; Suwannapong y col., 2010; Vidau y col., 2011). Sin embargo, otros estudios han encontrado una tasa de mortalidad menor en sus experimentos con abejas inoculadas en condiciones de laboratorio (Forsgren y Fries, 2010). Esta aparente discrepancia en los resultados podría deberse a las marcadas diferencias en los métodos de infección experimental utilizados por los distintos grupos de investigación (Higes y col., 2012a), en los cuales la procedencia y edad de las abejas, la dosis de esporas inoculada y las condiciones de conservación y purificación de las mismas difieren enormemente, no permitiendo la comparación objetiva entre resultados obtenidos.

Por otro lado, distintos estudios han demostrado la acción sinérgica entre este microsporidio y los pesticidas imidacloprida (Alaux y col., 2010), fipronil y tiacloprida (Vidau y col., 2011) a la hora de inducir una mayor mortalidad en las abejas infectadas de manera experimental y expuestas a dosis que resultan no letales para abejas no infectadas. Esta acción sinérgica entre el mencionado microsporidio y los pesticidas ha sido corroborada posteriormente en condiciones de campo, encontrándose una correlación positiva entre la exposición a dosis sub-letales de estas substancias y el recuento medio de esporas de *N. ceranae* por abeja (Pettis y col., 2012), así como un incremento en la susceptibilidad a la infección por parte de las abejas que se desarrollan en panales con residuos de pesticidas (Wu y col., 2012).

### b) en la colonia

La nosemosis tipo C muestra un largo período de incubación asíntomático en la colonia, lo que puede explicar la ausencia de síntomas evidentes antes de la muerte de la misma (Higes y col., 2008a). El recuento medio de esporas en abejas infectadas fluctúa ampliamente desde el principio al final de la enfermedad en abejas de interior, lo que demuestra que no es una medida fiable para estudiar la evolución de la nosemosis tipo C en las colonias. De hecho, la proporción de abejas infectadas ha mostrado ser el indicador más útil de la extensión de la enfermedad en la colonia, mientras que el recuento medio de esporas de pecoreadoras siempre ha sido un parámetro alternativo (Higes y col., 2008a; Meana y col., 2010). Asimismo, se ha detectado que incluso el tamaño de la muestra y el momento de recolección de la misma deben tenerse en cuenta a la hora de dar un diagnóstico fiable (Botías y col., 2012b).

Algunos de los rasgos clínicos de la infección, que normalmente pasan desapercibidos por los apicultores son un largo período de cría durante los meses fríos (incluso cuando normalmente debería ocurrir la parada invernal), un alto número de cuadros cubiertos por cría en relación a los cuadros cubiertos por abejas durante los meses templados, y bajas producciones de miel incluso uno o dos años antes del colapso. Finalmente, tras un periodo que varía entre 1,5-2 años, las colonias infectadas están claramente despobladas, presentando un descenso muy llamativo en la cantidad de abejas adultas, tras lo cual la colonia se muere (Higes y col., 2008a).

Figura 6. Aspecto de una colonia despoblada a consecuencia de la infección por *N. ceranae*.

Además, se han observado dos modos diferentes de colapso que podrían estar relacionados con la estación del año en el que las colonias mueren. Cuando el colapso sucede durante los meses fríos, más del 50% de las abejas muertas dentro de la colmena están infectadas, siendo el recuento medio de esporas en estas abejas siempre mayor de 10 millones, y las reinas (cuando se encuentran) también están infectadas. Por otro lado, cuando el colapso ocurre más tarde en primavera, el porcentaje de infección y media de recuento de esporas es más bajo. Sin embargo, bajo estas circunstancias las reinas normalmente no se encuentran infectadas. Probablemente, las diferencias entre estos dos casos reflejan la cantidad de abejas viejas y jóvenes en cada estación. De este modo, a comienzos de la primavera la proporción de abejas recién nacidas no infectadas reducirá los parámetros de infección, lo que a su vez, retrasará la infección de la reina (Higes y col., 2009a).

El debilitamiento de la colonia es probablemente debido al hecho de que las abejas fuertemente infectadas no regresan a la colmena y permanecen moribundas lejos de la colonia (Higes y col., 2008a, 2009a; Krajl y Fuchs, 2010). Debido a que el tamaño y número de individuos de una colonia de abejas melíferas depende directamente de la duración de vida de las obreras (Woyke, 1984; Khoury y col., 2011), y la infección con *N. ceranae* es capaz de acortar la esperanza de vida de las mismas (Higes y col., 2007; Paxton y col., 2007; Vidau y col., 2011) y de alterar su senescencia fisiológica (Dussaubat y col., 2009), esta enfermedad podría llevar a una disminución en la densidad poblacional de las colonias que contengan abejas infectadas. Esta hipótesis ha sido confirmada en condiciones de campo en varios estudios (Higes y col., 2008a; Soroker y col., 2011; Botías y col., 2010; 2012a), los cuales han encontrado una correlación negativa entre los niveles de infección por *N. ceranae* y la densidad de población de abejas adultas en las colmenas.

Por otro lado, en diversos estudios se ha demostrado una prevalencia mayor del parásito *N. ceranae* en abejas pecoreadoras en comparación con las abejas del interior de la colmena (más jóvenes), tanto en infecciones naturales (Meana y col., 2010; Martín-Hernández y col., 2012) como en infecciones experimentales (Smart y Sheppard, 2012). Por tanto, las abejas pecoreadoras estarían más expuestas a contraer la infección con *N. ceranae* y por tanto, a sufrir un acortamiento en su esperanza de vida. Como consecuencia de una alta tasa de mortalidad de las pecoreadoras, las abejas no infectadas podrían adelantar su maduración, produciéndose la transición de abeja de interior a pecoreadora de manera precoz, con el fin de compensar las pérdidas de pecoreadoras disponibles para mantener el equilibrio y adecuado desarrollo de la colmena (Huang y Robinson, 1996; Amdam y Omholt, 2003; Khoury y col., 2011). De este modo, el reparto de tareas en la colonia podría verse alterado (Wang and Moeller, 1970). Además, las abejas infectadas por *N. ceranae* también podrían sufrir un adelanto en su transición a las tareas de pecorea (Tofilski, 2009) y, mientras esto restauraría la proporción de pecoreadoras con respecto a la densidad de población, provocaría un descenso en la tasa de supervivencia de estas abejas (Neukirch, 1982; Schmid-Hempel y Wolf, 1988; Wolf y Schmid-Hempel, 1989), su efectividad y resiliencia como pecoreadoras (Oskay, 2007), y se reduciría el tiempo que cada abeja puede contribuir al crecimiento de la colmena, a la termorregulación y al cuidado de la cría entre otras tareas esenciales de la colmena. A su vez, la desatención de la cría, unida a la disminución en la

densidad poblacional, podría incrementar las probabilidades de desarrollar enfermedades de la cría como la ascosferosis provocada por el hongo *Ascosphaera apis* (Hedtke y col., 2011) en aquellas colonias afectadas por *N. ceranae*. Esta alteración en el desarrollo normal de las tareas de interior de la colonia también ha sido relacionada con una pérdida de efectividad en los tratamientos para el control de *Varroa* dependientes del contacto de las abejas con el producto acaricida y entre ellas en el interior de la colmena (Botías y col., 2012c), por lo que además, las colonias afectadas por nosemosis tipo C serían propensas a sufrir los efectos de la varroosis de manera más aguda. Consecuentemente, cuando la colonia llega al punto en el que la tasa de cría y renovación de la población es inferior a la tasa de pérdida de pecoreadoras en el campo debido a la infección por *Nosema*, la tasa de colapso de las colmenas también podría incrementarse (Khoury y col., 2011).

La alteración de la salud en la colonia también puede tener repercusiones en la productividad de la misma. La producción de miel está positivamente correlacionada con la densidad de población y cantidad de cría presente en las colmenas (Fries, 1988; Szabo y Lefkovitch, 1989), y por tanto, las colmenas con mayor población tienden a almacenar una mayor cantidad de miel (Eckert y col., 1994). Además, se ha demostrado que las pecoreadoras que provienen de colmenas altamente pobladas tienden a visitar más flores, y son más eficientes en sus tareas de pecorea (Wolf y Schmid-Hempel, 1990). Por ello, la disminución poblacional causada por la infección de *N. ceranae* en las colmenas podría llevar a la colmena a un estado subóptimo sanitario y productivo, hecho que ha sido confirmado en condiciones de campo (Botías y col., 2010; 2012a; Eischen y col., 2012).

Mientras que la nosemosis tipo A se ha considerado en general como un problema "menor", la nosemosis tipo C se considera un problema de sanidad mayor, tanto en la abeja individual (Paxton y col., 2007; Antúnez y col., 2009; Martín-Hernández y col., 2009) como en el conjunto de la colonia (Higes y col., 2008a; 2009b).

### 2.2.3. Epidemiología

Aunque los análisis retrospectivos realizados hasta el momento sugieren que *N. ceranae* ha estado en Europa desde la última década (Higes y col., 2006; Klee y col., 2007; Martín-Hernández y col., 2007; Paxton y col., 2007), la fecha exacta de su llegada a este continente sigue siendo desconocida, y su origen geográfico ha suscitado numerosos debates en la última década. Si bien la mayoría de los miembros de la comunidad científica acepta como válida la hipótesis sobre un origen "oriental" de este microsporidio y su consideración como agente patógeno exótico de entrada reciente en las poblaciones occidentales de *A. mellifera*. A pesar de ello, en la actualidad *N. ceranae* presenta una distribución mundial y altos niveles de prevalencia en varias regiones del mundo (Cox-Foxter y col., 2007; Huang y col., 2007; Klee y col., 2007; Martín-Hernández y col., 2007; Giersch y col., 2009; Tlak Gajger y col., 2010; Traver y Fell, 2011; Yoshiyama y Kimura, 2011).

En relación al patrón epidemiológico de la nosemosis tipo C, el rasgo más característico es que dicha infección se puede detectar a lo largo de todo el año en las colonias de abejas (en muestras recogidas en todas las estaciones y meses). En cuanto a la intensidad de la infección en cada estación del año, estudios realizados en climas templados sugieren que la cantidad de abejas infectadas en la colonia podría ser mayor en los meses primaverales (Oliver, 2011; Traver y Fell, 2011), mientras que en climas subtropicales y tropicales este máximo en la carga parasitaria de la colonia se alcanzaría en invierno (Chen y col., 2012). Aun así, los estudios sobre los patrones epidemiológicos de *N. ceranae* en las colonias infectadas coinciden en señalar la presencia constante de este microsporidio a lo largo del año. Esta falta de estacionalidad se une a un aumento de la muerte de las colonias manifestada por los apicultores, así como la ausencia de signos típicos de la nosemosis tipo A en colonias (Martín-Hernández y col., 2007; Tapaszki y col., 2009) como las diarreas y los abdómenes dilatados. De hecho, recientemente se ha sugerido que *N. ceranae* podría ser un factor clave de las pérdidas de colonias en colmenares profesionales en España (Higes y col., 2010).

En condiciones experimentales, se ha demostrado la transmisión horizontal de *N. ceranae* de abejas infectadas a reinas (Higes y col., 2009a) y entre abejas obreras (Smith, 2012) por medio del intercambio de alimento. Este hecho sugiere que la trofalaxia es una

importante vía de transmisión de abeja a abeja, y de colonia a colonia a través de la deriva. Sin embargo, en abejas infectadas y enjauladas se ha observado una menor inclinación para compartir el alimento con otras abejas, lo que podría afectar a este tipo de transmisión (Naug y Gibs, 2009).

A pesar de que la viabilidad de las esporas en la miel, el polen corbicular y la jalea real es todavía desconocida, se ha demostrado que estas matrices pueden actuar como reservorio de *N. ceranae* (Cox-Foster y col., 2007; Granato y col., 2008; Higes y col., 2008b; Giersch y col., 2009). El hecho de que haya esporas en el polen corbicular y en la miel puede deberse a la autocontaminación durante los procesos de recolección (regurgitación, saliva, etc) por un mecanismo desconocido hasta la fecha. Aunque la posibilidad de recoger esporas directamente de flores contaminadas no es descartable, esta opción parece altamente improbable (Higes y col., 2008b).

Los productos apícolas a su vez juegan un importante papel en la dispersión de esporas infectivas de *N. ceranae* de colmenar a colmenar a lo largo de diferentes áreas geográficas (Klee y col., 2007; Mutinelli y col., 2011). De este modo, el comercio de reinas se ha sugerido como fuente de infección en algunas áreas (Giersch y col., 2009).

Si bien es cierto que en la nosemosis tipo C no hay las marcas fecales típicas de la nosemosis tipo A (Higes y col., 2008a; 2010), y que los mecanismos de transmisión son diferentes, no se puede descartar la contaminación del material con esporas infectivas como camino más probable para el establecimiento de la infección en las colonias libres de infección (Van der Zee, 2010). Así, las abejas infectadas pueden contaminar el material apícola al ser machacadas durante el manejo apícola de las colmenas (Malone y col., 2001).

Hasta ahora, la mayor parte de los análisis de muestras parasitadas por *Nosema* spp. ha mostrado una alta tasa de detección de *N. ceranae* (Chauzat y col., 2007; Klee y col., 2007; Martín-Hernández y col., 2007; Tapazsi y col., 2009; Tlak Gagjer y col., 2010; Stevanovic y col., 2011), lo que se ha interpretado como un aparente desplazamiento de *N. apis*. Sin embargo, el corto periodo de tiempo de estudios en Europa (menor de cinco años), no permite confirmar tal hipótesis.

En cuanto a su rango de hospedadores, este microsporidio, a diferencia de *N. apis*, es capaz de parasitar otras especies de abejas diferentes a *A. mellifera* y *A. cerana*, como son *A. florea*, *A. dorsata* y *A. koschevnikovi* (Botías y col., 2009; Chaimanee y col., 2010) y otros himenópteros del género *Bombus* (Plischuk y col., 2009; Li y col., 2012).

Por otro lado, la reciente secuenciación del genoma de *N. ceranae* (Cornman y col., 2009) abre puertas a futuras investigaciones sobre este parásito.

### 2.2.4. Diagnóstico

Tanto el diagnóstico mediante examen macroscópico, como el microscópico y la identificación molecular se realizan del modo descrito para *N. apis* en el apartado 2.1.4.

### 2.2.5. Control

A pesar de que se han presentado alternativas para el tratamiento de la nosemosis tipo C, la fumagilina sigue siendo el antibiótico principalmente empleado para tratar patología al igual que en el caso de la nosemosis tipo A. Este antibiótico puede alcanzar un 100% de eficacia en las colonias infectadas por *N. ceranae* durante seis meses si es aplicado en una posología adecuada (Higes y col., 2008a, 2009). Además, la producción de miel en colonias infectadas y asintomáticas sufre una importante reducción cuando se comparan con colonias tratadas con fumagilina (Botías y col., 2010).

Hasta el momento, algunas de las sustancias que se han evaluado como alternativas a la fumagilina son ApiHerb® (Nanetti, 2009), Nozevit® (Tlak Gajger y col., 2009), Nonosz® (Békési y col., 2009), ácido oxálico (Higes y col., 2012b) o lisozimas de procedencia animal y aceites esenciales de la planta *Vetiveria zizanioides* (Maistrello y col., 2009). De entre las moléculas probadas destacan el resveratrol (fitoalexina natural; trans-3,5,4'-trihydroxystilbeno) y el timol (terpeno; 2- iso propil-5-metilfenol), los cuales mostraron capacidad para disminuir la carga parasitaria, y como consecuencia la tasa de mortalidad, en abejas infectadas experimentalmente (Maistrello y col., 2009; Costa y col., 2010). Por otro lado, la utilización de metabolitos bacterianos como la surfactina (Porrini y col., 2010) o de

ARN de doble hebra homólogo a los genes que codifican para el complejo ADP/ATP en *N. ceranae* (Paldi y col., 2010) han mostrado asimismo un efecto inhibidor de la proliferación de este parásito en las abejas infectadas de manera experimental. Todos estos tratamientos alternativos, a pesar de presentarse como opciones prometedoras y alternativas al uso de la fumagilina, requieren de una confirmación de eficacia en condiciones de campo y de un estudio sobre los excipientes más apropiados con los que aplicar estos compuestos activos, lo cual debería abordarse en futuros estudios.

A su vez, los estudios orientados a la profilaxis y prevención de la enfermedad sugieren que tanto la fumigación con ácido acético como la aplicación de calor (49 ± 1 °C durante 24 horas) o la irradiación con un haz de electrones son prácticas eficaces en la desinfección del material apícola a corto plazo (Pernal y col., 2010), por lo que éstas deberían efectuarse periódicamente en las colonias de abejas.

# CAPÍTULO I

## Patrón epidemiológico de las nosemosis en las colonias de *A. mellifera* y factores relacionados

Las nosemosis en las abejas melíferas han sido causadas tradicionalmente por el microsporidio *N. apis*. Sin embargo, como ya se ha comentado anteriormente, *N. ceranae* ha sido descubierto infectando a las abejas melíferas europeas *A. mellifera* en años relativamente recientes. Tras este hallazgo, la mayor parte de los análisis de muestras parasitadas por *Nosema* spp. ha mostrado una mayor prevalencia de *N. ceranae* con respecto a *N. apis* en diversas regiones del mundo. En el Artículo 1 de esta memoria se analizan los patrones epidemiológicos presentados por *N. ceranae* y *N. apis* en colonias de abejas. Para ello se examinó de forma comparada la prevalencia de ambas especies de microsporidios tanto en colonias como en apiarios en los que se había detectado la infección por ambas especies.

Por otro lado, la rápida difusión de *N. ceranae* por todas las zonas geográficas donde se practica la apicultura ha sido uno de los enigmas que más debate ha generado entre científicos y apicultores. Si bien, a pesar de que el origen geográfico de *N. ceranae* está por determinar, la mayoría de los miembros de la comunidad científica aceptan como válida la hipótesis de un origen "oriental" de este microsporidio, y su consideración como patógeno exótico en muchos países de occidente, mientras que otros grupos de investigación argumentan que se trata de un patógeno endémico en occidente. Sin embargo, esta segunda opción descarta su reciente distribución mundial y su incremento de prevalencia en los diferentes continentes de la que no se tenía constancia previa hasta los primeros años del siglo XXI. Por ello, en esta memoria también se evalúa la hipótesis sobre una reciente y gradual invasión de *N. ceranae* en las poblaciones occidentales de abejas melíferas a través de dos ensayos diferentes. Así, en el Artículo 2 se analiza la hipótesis de la entrada reciente de este microporidio en nuestro país y el consiguiente aumento de su prevalencia en las poblaciones de abejas ibéricas, lo cual podría aportar evidencias sobre el momento de entrada del microsporidio en nuestro país. Para ello fueron analizadas muestras históricas de miel comercial almacenadas en el Laboratorio de la Miel del Centro Apícola Regional (CAR) desde el año 1988 hasta el 2009, con el fin de identificar la miel más antigua en la que se detectaran esporas de *N. ceranae*, así como determinar la prevalencia de este microsporidio, en cada uno de los años examinados. Se utilizó la miel como un marcador de la epidemiología real de este agente patógeno esporulado en las colonias

de abejas en el campo. Asimismo, el incremento gradual en la presencia de *N. ceranae* en las colonias de abejas españolas se compara con la prevalencia mostrada en condiciones de campo tras el análisis de muestras de abejas obreras recogidas en colonias de distintos años y orígenes geográficos. En segundo lugar, en el Artículo 3 se evalúa la hipótesis sobre el origen asiático *N. ceranae* y de su hospedador originario. Esto además podría confirmar la hipótesis sobre la condición de hospedador reciente sugerida para *A. mellifera*. Para ello se buscaron pruebas sobre la amplia distribución de este microsporidio en poblaciones de diversas especies del género *Apis* procedentes de latitudes orientales, así como sobre la existencia de signos sobre su incipiente invasión del hospedador *A. mellifera* fuera de Asia. Además, en este estudio se aporta la primera cita sobre la presencia de *N. ceranae* en la especie asiática *A. koschevnikovi*. Asimismo, este microsporidio se detecta por primera vez en poblaciones de *A. cerana* en Indonesia y Corea del Sur, y tanto en *A. cerana* como en *A. mellifera* en las Islas Salomón, países en los que hasta el momento no se había encontrado este patógeno.

En este primer capítulo de la memoria también se evalúan algunos de los posibles factores responsables de los patrones epidemiológicos observados para *N. ceranae* y *N. apis*, los cuales pueden estar relacionados con el hospedador, con el parásito o con el medio ambiente. En el caso del hospedador, el Artículo 4 aporta evidencias sobre la capacidad de *N. ceranae* para invadir un amplio rango de hospedadores, presentándose un estudio en el cual se analiza la posible presencia de este microsporidio en himenópteros nativos de Argentina del género *Bombus*. Además, en el Artículo 5 se añaden más pruebas sobre la amplia distribución de *N. ceranae* en poblaciones de *A. mellifera*, tras analizar la posible presencia del citado microsporidio en muestras de abejas de la subespecie norteafricana *A. mellifera intermissa* procedentes de colonias colapsadas de Argelia.

En cuanto a los factores relacionados con el parásito, se presentan dos estudios en los cuales se compara el potencial biótico de ambos microsporidios expuestos a distintas condiciones de temperatura. El Artículo 6 examina las posibles diferencias en el ciclo biológico de *N. ceranae* y *N. apis* en las abejas infectadas experimentalmente y mantenidas en condiciones controladas de laboratorio, mientras que el Artículo 7

compara el desarrollo de ambos microsporidios en condiciones de temperaturas crecientes.

Por último, también existen factores medioambientales que pueden tener un impacto en los patrones epidemiológicos observados para las nosemosis en nuestro país. De este modo, en el Artículo 8 se valora uno de los mecanismos de dispersión y transmisión de los microsporidios *N. ceranae* y *N. apis* desde las colonias de abejas infectadas hacia aquellas libres de infección. Para ello se estudió el papel potencial de las egagrópilas de abejaruco (*Merops apiaster* L., Meropidae) como fómite de esporas infectivas de estos patógenos. Los abejarucos son aves insectívoras cuya presa predominante es la abeja melífera y son, por tanto, un visitante habitual de los apiarios tanto durante su etapa de cría, como durante su etapa de migración entre el continente africano y el continente europeo. Es por ello que las egagrópilas regurgitadas por estos pájaros se componen mayoritariamente de los exoesqueletos de las abejas previamente ingeridas, fundamentalmente pecoreadoras, algunas de ellas posiblemente infectadas por los microsporidios *N. ceranae* y/o *N. apis*. Debido a que las esporas de estos microporidios son relativamente resistentes a las condiciones ambientales, el papel de las egagrópilas como reservorio y vector de transmisión de los mismos se presenta como un posible factor clave en la dinámica de propagación de las nosemosis dada la etología del ave.

# Artículo 1

## Infecciones de microsporidios en *Apis mellifera*: coexistencia o competición. ¿Está *Nosema ceranae* reemplazando a *Nosema apis*?

Raquel Martín-Hernández, Cristina Botías, Encarna Garrido-Bailón, Amparo Martínez-Salvador, Lourdes Prieto, Aránzazu Meana y Mariano Higes

*Environmental Microbiology (2012) 14(8): 2127-2138*

*Desde hace algunos años se ha sugerido un reemplazo de* Nosema apis *por parte de* Nosema ceranae *en algunas poblaciones de colonias de abejas melíferas. Sin embargo, este reemplazo de un microsporidio por el otro no se ve apoyado por los datos de distribución y prevalencia de ambos en colmenares profesionales españoles (estudio transversal), ni por el patrón estacional en colmenas experimentales en las que existe una co-infección, ni por los datos de prevalencias en abejas analizadas individualmente dentro de cada colonia (tanto en obreras como en zánganos). No obstante,* N. ceranae *ha mostrado una mayor prevalencia en todas las escalas estudiadas, lo que podría indicar ciertas ventajas en el desarrollo de este microsporidio con respecto a* N. apis*, o de otro modo, que éste se encuentra más adaptado a las condiciones del territorio español. Además, ambos microsporidios muestran un patrón de preferencias por condiciones ambientales diferentes, teniendo en cuenta la prevalencia que cada uno de ellos muestra para los distintos pisos bioclimáticos estudiados dentro del territorio español.*

*Finalmente, todos los análisis fueron llevados a cabo utilizando un control interno de PCR (IPC), lo cual garantiza la fiabilidad de los datos extraídos de los análisis de PCR. Este IPC proporciona una herramienta útil para la detección laboratorial de los patógenos de la abeja melífera.*

***PRESENTACIONES EN CONGRESOS:***

*Martín-Hernández R., Garrido-Bailón E., Botías C., Meana A., Prieto L., Higes M. Rapid diagnostic method for* Nosema *spp. detection using the honeybee COI as internal control in a Triplex PCR. Simposio: Diagnosis and Control of Bee Diseases, 26-28 de Agosto 2008, Friburgo (Alemania).*

*Martín-Hernández R., Botías C., Meana A., Higes M. Nosemosis Diagnostic. COLOSS Workshop:* Nosema *disease, lack of knowledge and work standardization. 19-22 de Octubre 2009, Guadalajara (España).*

*Martín-Hernández R., Botías C., Meana A., Higes M. Prevalence of* N. apis *and* N. ceranae *in colonies with mixed infection . 4º Congreso EurBee, 7-9 de Septiempre 2010, Ankara (Turquía).*

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# Microsporidia infecting *Apis mellifera*: coexistence or competition. Is *Nosema ceranae* replacing *Nosema apis*?

**Raquel Martín-Hernández,[1,2]* Cristina Botías,[1] Encarna Garrido Bailón,[1] Amparo Martínez-Salvador,[3] Lourdes Prieto,[4] Aránzazu Meana[5] and Mariano Higes[1]**

[1]*Bee Pathology laboratory, Centro Apícola Regional, JCCM, 19180 Marchamalo, Spain.*

[2]*Instituto de Recursos Humanos para la Ciencia y la Tecnología (INCRECYT), Fundación Parque Científico y Tecnológico de Albacete, Spain.*

[3]*Epidemiology Consultant, C/Puente la Reina, 28050 Madrid, Spain.*

[4]*Instituto Universitario de Investigación en ciencias policiales (IUICP), Comisaría General de la Policía Científica, DNA Laboratory, Madrid, Spain.*

[5]*Animal Health Department, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.*

## Summary

***Nosema ceranae* has been suggested to be replacing *Nosema apis* in some populations of *Apis mellifera* honeybees. However, this replacement from one to the other is not supported when studying the distribution and prevalence of both microsporidia in professional apiaries in Spanish territories (transverse study), their seasonal pattern in experimental hives with co-infection or their prevalence at individual level (either in worker bees or drones). Nevertheless, *N. ceranae* has shown to present a higher prevalence at all the studied levels that could indicate any advantage for its development over *N. apis* or that it is more adapted to Spanish conditions. Also, both microsporidia show a different pattern of preference for its development according to the prevalence in the different Spanish bioclimatic belts studied. Finally, the fact that all analyses were carried out using an Internal PCR Control (IPC) newly developed guarantees the confidence of the data extracted from the PCR analyses. This IPC provides a useful tool for laboratory detection of honeybee pathogens.**

Received 16 June, 2011; revised 11 October, 2011; accepted 23 October, 2011. *For correspondence. E-mail rmhernandez@jccm.es; Tel. (+34) 949250026; Fax (+34) 949250176.

## Introduction

Nosemosis is the most widespread of adult bee diseases, causing significant economic losses to bee-keepers (OIE, 2008; Giersch *et al*., 2009; Heintz *et al*., 2011). To date, two microsporidia species have been associated with this disease, both infecting honeybees worldwide: *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries *et al*., 1996). The first of these, *N. apis*, was shown to infect the honeybee *Apis mellifera* more than 100 years ago and it was one of the first microsporidia to be described. By contrast, *N. ceranae* was only recently shown to infect *A. mellifera*, both in Europe and Asia (Higes *et al*., 2005; 2006; Huang *et al*., 2007).

*Nosema ceranae* was initially described in *Apis cerana* (Fries *et al*., 1996) and the expansion of its host range to *A. mellifera* probably occurred in the last decade of the 20th Century (Klee *et al*., 2007). Nosemosis caused by *N. ceranae* is now considered to be an internationally emergent disease (Higes *et al*., 2010). Moreover, the rise in microsporidian infection of honeybees observed worldwide during the last 15 years (Klee *et al*., 2007; Martín-Hernández *et al*., 2007; Calderón *et al*., 2008; Williams *et al*., 2008; Giersch *et al*., 2009; Invernizzi *et al*., 2009; Higes *et al*., 2010; Yoshiyama and Kimura, 2011) is strongly associated with the colonization of this new host by *N. ceranae* (Martín-Hernández *et al*., 2007), which is currently a more common infection than that of *N. apis* in European honeybees (Chen and Huang, 2010).

Epidemiological differences between these two microsporidia could support the rise of *N. ceranae* over *N. apis*. For example, *N. ceranae* seems to be less host-specific since it has been found in a wider range of hosts, including *Apis koschevnikovi* (Botías *et al*., 2009), *Apis florea* (Suwannapong *et al*., 2010a,b), *Apis dorsata* (Chaimanee *et al*., 2010) and some Argentinean bumble bee species (Plischuk *et al*., 2009), in which *N. apis* has been never reported. Moreover, differences have been found related to the season when infection can be detected. In this sense, *N. apis* infections have been associated with temperate climates and seasonal patterns, hardly being detected during the summer but with a small peak in the autumn and a stronger peak in spring (Bailey, 1955; Hornitzky, 2005). By contrast, *N. ceranae* seems not to have this seasonality, infecting bees

**Table 1.** Sensitivity of the method for the detection of *Nosema* spp.

| | A | | | | | | | | B | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bee macerate | | | | Purified spores | | | | Bee macerate | | Purified spores | |
| Replicate id. | 1–1 | 1–2 | 2–1 | 2–2 | 1–1 | 1–2 | 2–1 | 2–2 | 2–1 | 2–2 | 2–1 | 2–2 |
| *N. ceranae* | 2.5 | 2.5 | 2.5 | 2.5 | 2500 | 1000 | 50 | 25 | 2.5 | 2.5 | 250 | 25 |
| *N. apis* | 10 | 50 | 10 | 5 | 250 | 250 | 250 | 250 | 5 | 25 | 500 | 250 |

Limits of detection in Triplex PCR: values equivalent to number of spores diluted in 150 μl (counted either from bee macerates or purified spores) detected by PCR. (A) DNA template volume added per reaction was either 5 μl of *N. apis* or 5 μl of *N. ceranae*. Two dilution replicates and two extraction replicates. (B) DNA template volume added per reaction was 5 μl of *N. apis* + 5 μl of *N. ceranae*. Two replicates.

throughout the year at some latitudes (Martín-Hernández *et al*., 2007; Giersch *et al*., 2009; Tapaszti *et al*., 2009). This could be related to the higher biotic potential of *N. ceranae* at different temperatures when compared with *N. apis* (Martín-Hernández *et al*., 2007; 2009).

Indeed, it was recently suggested that *N. ceranae* is replacing *N. apis* in some populations of European honeybees. This statement is consistent with descriptions that report such a displacement between microsporidia infecting the same host (reviewed by Solter *et al*., 2002; Solter and Becnel, 2003; Vizoso and Ebert, 2005). In fact, co-infecting parasites are likely to compete for the same resources. In genetically diverse infections, those with genotypes that employ different strategies to exploit common resources are expected to compete for these (Ebert *et al*., 2000). However, although there are data supporting the replacement of *N. apis* by *N. ceranae* in *A. mellifera* (Klee *et al*., 2007; Chen *et al*., 2008; Botías *et al*., 2011; Yoshiyama and Kimura, 2011), this is not so clear in other countries/regions (Gisder *et al*., 2010). In fact, the potential inter-species competition between both these microsporidia in the *A. mellifera* host has not been studied in depth.

To study parasite interactions and the relationship of these with their host, considering different ecosystems can be established. In the first place, one could assess the exterior environment in which honeybees live, forage and establish relationships with other bee communities. The hive can be considered as a second level, composed of thousands of individuals that live and develop in the same space. As a third and final level, we could focus on the individual bees (belonging to different castes as workers and drones), where two different pathogens may coexist (or compete) in a single environment. Accordingly, the aim of this work was to study the complex '*N. apis*/*N. ceranae*/honeybee' relationship at different levels.

Additionally, to carry out this study, we developed and used an improved PCR assay to detect *Nosema* sp., based on that described previously (Martín-Hernández *et al*., 2007) but incorporating the use of an Internal PCR Control (IPC), detecting the mitochondrial Citochrome Oxidase subunit I gene (COI) of *A. mellifera*, which is always co-extracted in samples (infected or not infected) and therefore is a good internal control in the amplification to avoid false negative results.

## Results

### *Triplex PCR*

Primers were designed to amplify the COI gene of *A. mellifera* as an internal control in the standard PCR assay to identify *Nosema* species: COI-F (5′-GGGTCCAAGACCAGGAACTGGAT-3′) and COI-R (5′-GCGCGGAAATTCCTGATATATGAAGAGAAAA-3′). In each of the primers, GC tails (underlined) were added to augment the annealing temperature and thereby enhance their specificity. All the PCR products amplified with these primers were of the expected size (119 bp) and when the sequences of the three PCR products obtained were compared by BLAST (NCBI database), they corresponded to *A. mellifera* sequences or those of Hymenoptera (undetermined species sequences).

Primers showed a satisfactory annealing temperature from 57.1°C to 66.1°C but, since the aim of this work was to combine them in the Triplex PCR amplification with 321-APIS and 218-MITOC, all the studies here presented were made at 61.8°C. As well, the COI primers were used at a concentration of 0.03 μM, which produced a clear (albeit weak) band without reducing the sensitivity of the other two pairs of primers. Thus, the sensitivity of detecting *Nosema* spp. was not affected by the use of the COI primers in the Triplex PCR. When the sensitivity of the Triplex PCR methodology was tested (Table 1), the limit of detection for both *N. ceranae* and *N. apis* in bee macerates was always lower than in purified spores. The reliability of the limit of detection was very good for both microsporidia when analysed in bee macerates and for *N. apis* analysed in purified spores. However, the limits of detection for *N. ceranae* in purified spores were much more variable; the higher level of detection found only for the called '*N. ceranae* purified 1', probably is due to an inaccurate pipetting when preparing the mother solution.

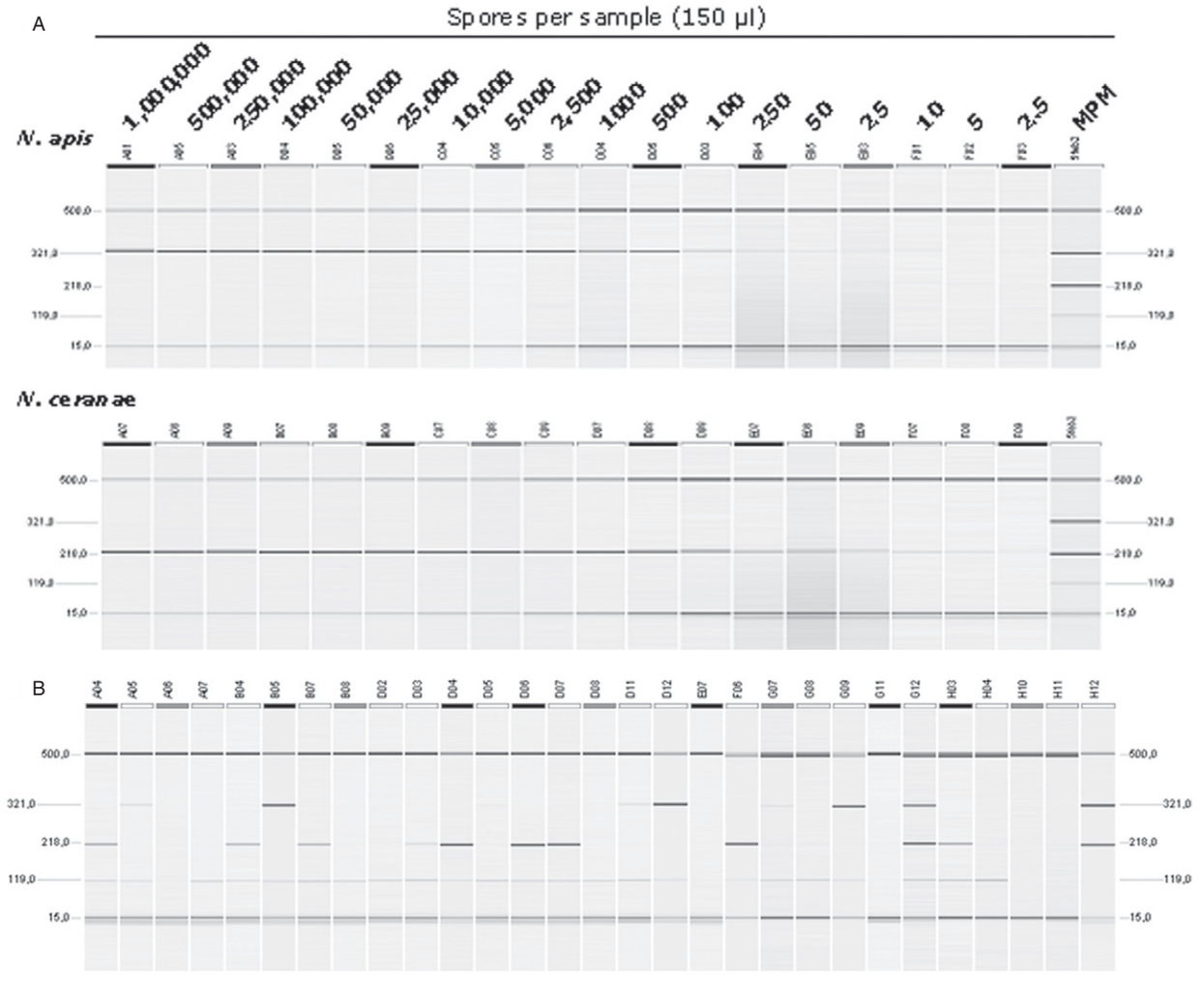


**Fig. 1.** Capillary electrophoresis of amplicons obtained by Triplex PCR for *Nosema* spp. analysis. COI (IPC) size 119 bp, *N. ceranae* size 218–219 bp, *N. apis* 321 bp. Alignment marker 15–500 bp.
A. Sensitivity and specificity of *Nosema* spp. analysed in triplex PCR. Lack of COI band resolution is attributable to the combined effect of the suboptimum COI primers concentration used and to the samples dilution to determine the sensitivity of the technique.
B. Results on clinical samples. *Nosema apis* positive lanes: A05, B05, D11, D12, G07, G09; *N. ceranae* positive lanes: A04, B04, B07, D03, D04, D06, D07, F06, H03, G08; mixed infection (*N. apis* + *N. ceranae*) positive lanes: G12, H12; *Nosema* spp. negative lanes: A07, B08, D02, D05, D08, H04; blanks (including negative controls of extraction and PCR): A06, E07, G11, H10, H11.

A high concentration of *N. apis* or *N. ceranae* spores was sometimes associated with the poor resolution of the COI amplicon. However, this product was always detected even when no amplification of *Nosema* sp. was evident. Thus, the extraction protocol employed appears to work correctly.

The sensitivity of the triplex PCR was very high and the amplification of the products of the *Nosema* sp. was not impaired by the introduction of the COI primers (Fig. 1). The IPC was clearly seen when samples are negatives to *Nosema* spp., confirming the DNA extraction procedure was accurate, even when a single bee was tested. Indeed, the sensitivity of *Nosema* detection was higher than that reported previously using the same pair of primers without the internal control (Martín-Hernández *et al.*, 2007), although this difference is most probably due to the different polymerases used: Faststart vs. HighFidelity (both Roche Diagnostic).

### *Transverse study: prevalence of* Nosema *in Spain*

The most prevalent microsporidia infecting honeybees in Spain was *N. ceranae* (Table 2). This species was found in over 40% of the colonies sampled in the two years studied (2006–2007), both in spring and autumn, and with no differences between any of the periods studied ($\chi^2$;

**Table 2.** Prevalence of *N. apis* and *N. ceranae* in Spain in the transverse study.

| | | *N. apis* | | *N. ceranae* | | Co-infection *N. apis* + *N. ceranae* | |
|---|---|---|---|---|---|---|---|
| | % | Prevalence | 95% CI | Prevalence | 95% CI | Prevalence | CI 95% |
| 2006 | Spring | 14.6 | 11.8–17.5 | 46.4 | 42.4–50.4 | 6.5 | 4.5–8.5 |
| | Autumn | 10.3 | 7.4–13.3 | 42.5 | 37.9–47.2 | 4.8 | 2.8–6.9 |
| 2007 | Spring | 8.2 | 5.7–10.6 | 47.3 | 43.0–51.7 | 4.4 | 2.5–6.2 |
| | Autumn | 11.2 | 7.7–14.8 | 43.5 | 38.0–49.0 | 5.5 | 2.9–8.1 |

$P > 0.05$). By contrast, *N. apis* was less prevalent and it was never found at a prevalence above 15%. Moreover, the prevalence of this species in spring 2006 was significantly higher than in spring 2007 ($\chi^2$; $P < 0.05$), although it was similar in autumn of both years ($\chi^2$; $P > 0.05$). Co-infection of both microsporidia was also observed but always below 7% and with a similar prevalence in all the seasons sampled ($\chi^2$; $P > 0.05$).

Regarding the distribution of *Nosema* sp. in Spain (Fig. 2), *N. ceranae* prevalence was significantly higher ($\chi^2$; $P < 0.05$) in hotter areas of Spain (mesomediterranean and termomediterranean bioclimatic belts) than in warmer or colder ones (Table 3A). On the contrary, *N. apis* seems to follow an descending gradient of prevalence (Table 3B) and the lower presence was found in the bioclimatic belts called 'colline' and the Termomediterra-

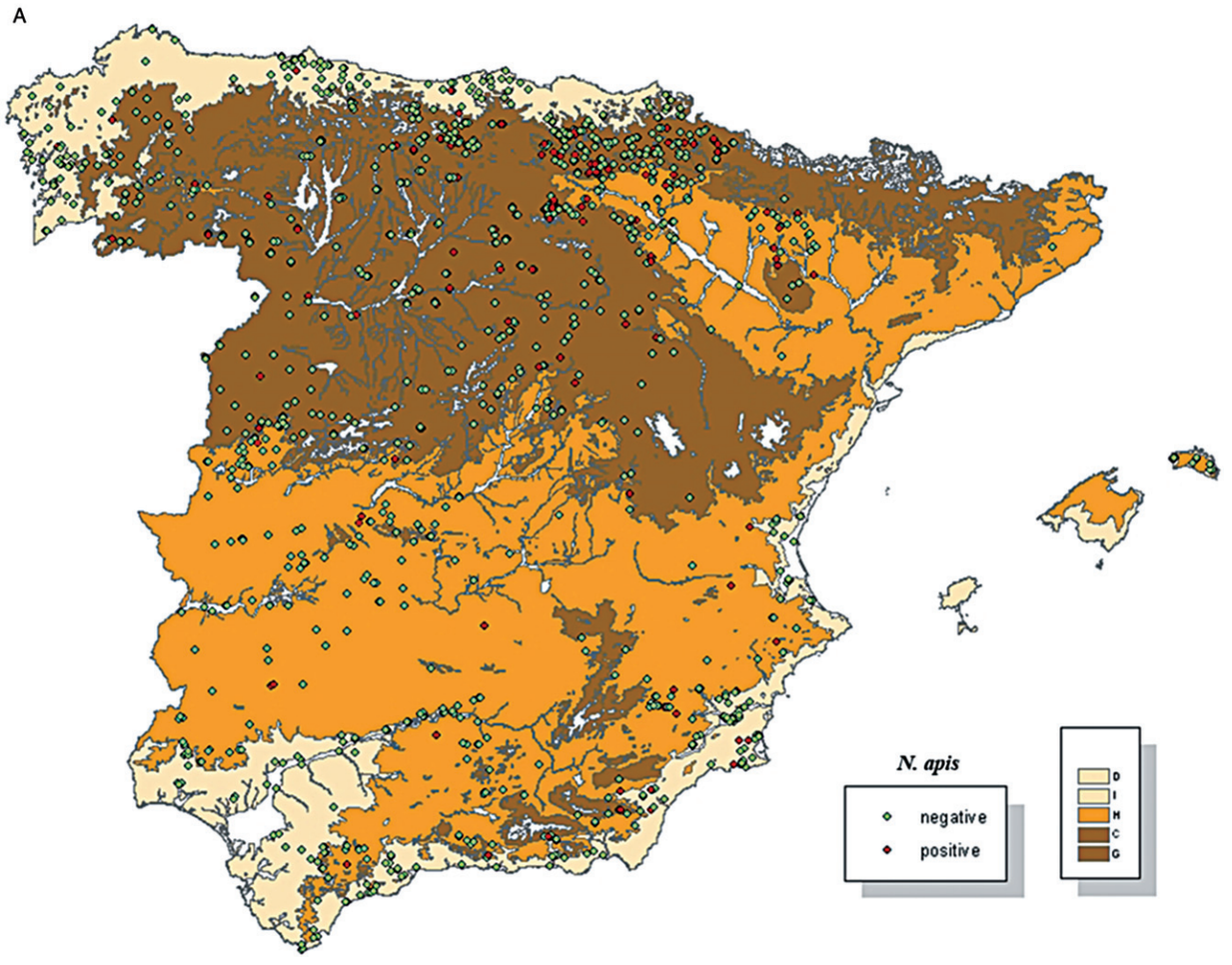


**Fig. 2.** Transverse study: maps showing the distribution of samples positives to *N. apis* (A) or *N. ceranae* (B) in Spain, according to the bioclimatic belts described by Rivas-Martínez (1987): Montane (C), Colline (D), supramediterranean (G), mesomediterranean (H), termomediterranean (I).

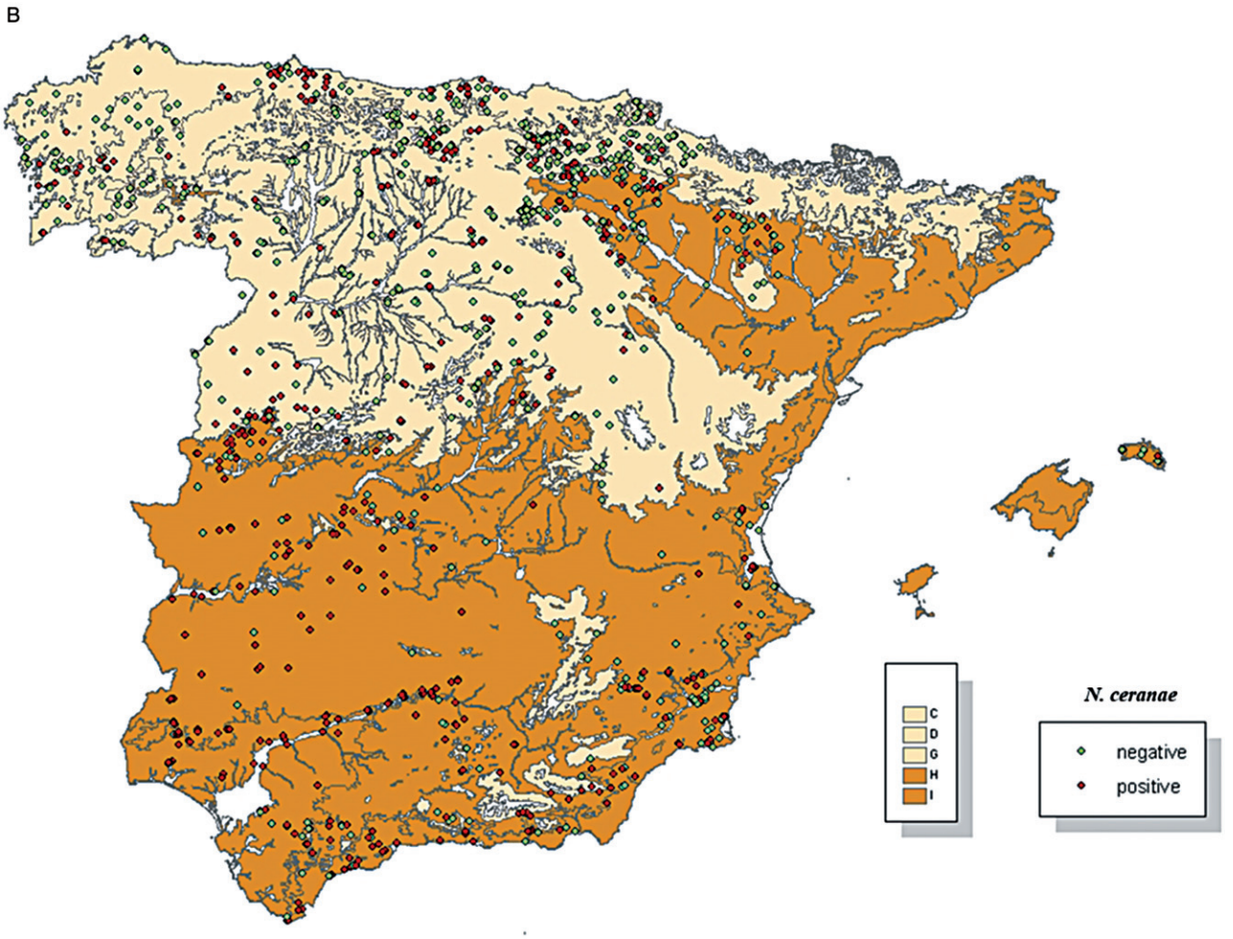


**Fig. 2.** *cont.*

nean which correspond with the areas with the lower number of months when frosts are possible (6 or 3 months per year respectively). The prevalence was increasing to the milder areas (Montane and Supramediterranean bioclimatic belts) but where the period of the year when frosts are possible are higher (both 10 months per year).

*Longitudinal study: seasonal influences*

A total of 408 samples from 30 honeybee colonies were analysed to assess whether seasonal differences in *Nosema* infection were evident. The most prevalent microsporidia was *N. ceranae*, which once detected in colonies almost always persisted throughout the study (Fig. 3). While on most occasions *N. ceranae* was found alone, sometimes co-infection with *N. apis* was detected. By contrast, *N. apis* was only observed alone in four colonies and in just four different samples out of 408 analysed. *Nosema apis* (alone or together with *N. ceranae*) was more frequently found at specific times of the year, between March and June, and from September to November (coinciding with the Spanish Spring and Autumn). It was not detected in Apiary 1 during the other sampling months and in Apiary 2, *N. apis* was infrequently detected from January to February, or July to August (Fig. 3).

*Individual level*

A total of 781 worker honeybees (400 interior bees and 381 forager bees) were analysed individually (Table 4), in which the most prevalent microsporidia was *N. ceranae* (29.96%). This *Nosema* sp. was always detected in both house and forager bees, although it was more frequent in foragers (39.63%) than in house bees (20.75%). Conversely, *N. apis* was found very infrequently in the bees (0.9%) and it was only detected in forager bees (1.8%). honeybees infected with both microsporidia were found occasionally (0.6%) either in interior bees (0.25%) or forager bees (1.05%). Drone bees were infected by both *Nosema* spp. (Table 4) too, and again *N. ceranae* was

**Table 3.** *Nosema* spp. distribution among the bioclimatic belts (A: *N. ceranae*; B: *N. apis*) according to Rivas-Martínez (1987) classification.

A

| | | | *N. ceranae* | | | | | |
|---|---|---|---|---|---|---|---|---|
| Bioclimatic belt | Climatic characteristics | *n* | Positive | Prevalence (%) | $\chi^2$ | *P* | $\chi^2$ | *P* |
| Montane (C) | T 12 to 6°C, m 2 to −4°C, M 10 to 3°C, IT 240 to 50, H: IX-VI. Sub-humid to hyper-humid | 301 | 105 | 34.9 | – | – | | |
| Supramediterranean (G) | T 13to 8°C, m −1 to −4°C, M 9 to 2°C, IT 210 to 60, H: IX-VI. Semi-arid to hyper-humid | 454 | 171 | 37.7 | 0.60 | 0.4371[a] | – | – |
| Colline (D) | T > 12°C, m > 2°C, M > 10°C, IT > 240, H: XI-IV. Sub-humid to hyper-humid | 191 | 80 | 41.9 | 2.44 | 0.1181[a] | 1.01 | 0.3156[b] |
| Mesomediterranean (H) | T 17 to 13°C, m 4 to −1°C, M 14 to 9°C, IT 350 to 210, H: X-IV. Semi-arid to hyper-humid | 492 | 274 | 55.7 | 32.40 | **0.0000**[a] | – | – |
| Termomediterranean (I) | T 19 to 17°C, m 10 to 4°C, M 18 to 14°C, IT 470 to 350, H: XII-II. Arid to humid | 256 | 145 | 56.6 | 139.81 | **0.0000**[a] | 0.06 | 0.8039[c] |
| Missing | | 137 | | | | | | |
| Total | | 1831 | 834 | 45.5 | | | | |

**a.** Prevalence of *N. ceranae* on each bioclimatic belt was compared with the lowest level (Montane, C).
**b.** Prevalence of *N. ceranae* on Colline (D) was compared with Supramediterranean (G).
**c.** Prevalence in Termomediterranean (I) belt compared with Mesomediterranean (H) belt.
Bold indicates statistical significance.
T: annual average temperature. m: average of minim temperature on the colder month. M: average of maximum temperatures on the colder month. IT: Termic index = (T + m + M) × 10. H: months (in Roman numbers) when frost is statistically probable to happen. Ombroclimate classification according to rainfall: arid < 200 mm, semi-arid 200–350 mm, dry 350–600 mm, sub-humid 600–1000 mm, humid 1000–1600 mm, hyper-humid > 1600 mm.

B

| | | | *N. apis* | | | | | |
|---|---|---|---|---|---|---|---|---|
| Bioclimatic belt | Climatic characteristics | *n* | Positive | Prevalence (%) | $\chi^2$ | *P* | $\chi^2$ | *P* |
| Colline (D) | T > 12°C, m > 2°C, M > 10°C, IT > 240, H: XI-IV. Sub-humid to hyper-humid | 191 | 6 | 3.14 | – | – | | |
| Termomediterranean (I) | T 19 to 17°C, m 10 to 4°C, M 18 to 14°C, IT 470 to 350, H: XII-II. Arid to humid | 256 | 16 | 6.25 | 2.26 | 0.1328[a] | | |
| Mesomediterranean (H) | T 17 to 13°C, m 4 to −1°C, M 14 to 9°C, IT 350 to 210, H: X-IV. Semi-arid to hyper-humid | 492 | 48 | 9.76 | 8.27 | **0.0040**[a] | | |
| Montane (C) | T 12 to 6°C, m 2 to −4°C, M 10 to 3°C, IT 240 to 50, H: IX-VI. Sub-humid to hyper-humid | 301 | 47 | 15.61 | 18.91 | **0.0001**[a] | – | – |
| Supramediterranean (G) | T 13to 8°C, m −1 to −4°C, M 9 to 2°C, IT 210 to 60, H: IX-VI. Semi-arid to hyper-humid | 454 | 79 | 17.40 | 23.89 | **0.0000**[a] | 0.42 | 0.5192[b] |
| Missing | | 137 | | | | | | |
| Total | | 1831 | 205 | 11.20 | | | | |

**a.** Prevalence of *N. apis* on each bioclimatic belt was compared with the lowest level (Colline, D).
**b.** Prevalence of *N. apis* on Supramediterranean (G) belt was compared with Montane (C) belt.
Bold indicates statistical significance.
T: annual average temperature. m: average of minim temperature on the colder month. M: average of maximum temperatures on the colder month. IT: Termic index = (T + m + M) × 10. H: months (in Roman numbers) when frost is statistically probable to happen. Ombroclimate classification according to rainfall: arid < 200 mm, semi-arid 200–350 mm, dry 350–600 mm, sub-humid 600–1000 mm, humid 1000–1600 mm, hyper-humid > 1600 mm.

more prevalent and co-infection was more frequently found in this caste than in workers.

## Discussion

In this work, we focused on three different levels of study: (i) the prevalence and distribution of *Nosema* throughout Spain; (ii) the colonies where both *Nosema* species co-habited; and finally as the lowest level (iii) the individual honeybees from colonies where both species have been found.

When infection by *Nosema* sp. was studied on the country scale, the mean prevalence of *N. ceranae* in spring and autumn over the 2 year period of sampling was around 50% (alone or in co-infection), displaying a wider dispersion and a higher prevalence than *N. apis*. This high prevalence of *N. ceranae* seems to be frequent worldwide (Chauzat *et al.*, 2007; vanEngelsdorp *et al.*, 2009; Traver and Fell, 2011). Conversely, *N. apis* was less prevalent, found in about 20% of all the samples tested, as described elsewhere (Pajuelo and Fernández Arroyo, 1979; Orantes Bermejo and García-Fernández, 1997).

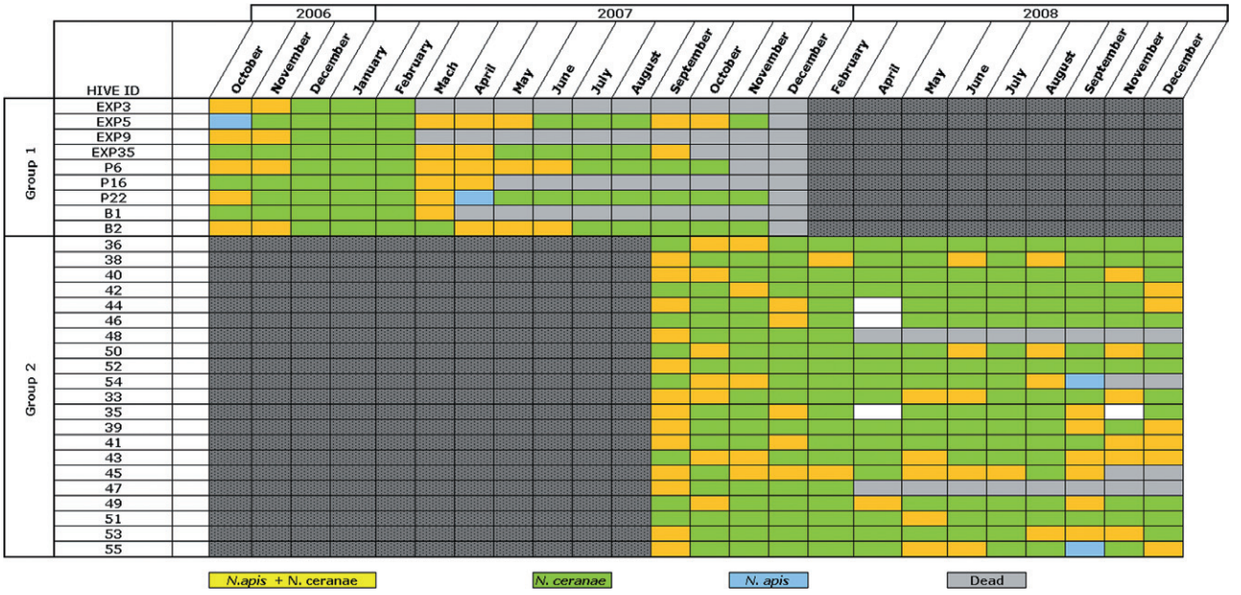


**Fig. 3.** Longitudinal study: natural dynamics of *Nosema* sp. infection in *N. apis* and *N. ceranae* co-infected colonies.

These data could support the hypothesis that the colonization of *A. mellifera* due to the spread of *N. ceranae* may be responsible for the rise in the *Nosemosis* detected in this country (Martín-Hernández *et al*., 2007). However, this situation is not universal because in Germany, *N. apis* has been reported to be more prevalent than *N. ceranae* (Gisder *et al*., 2010). Indeed, the difference in prevalence declared in distinct countries is remarkable since in some cases, the prevalence of *N. ceranae* is considered to be very high (Chauzat *et al*., 2007; vanEngelsdorp *et al*., 2009; Bacandritsos *et al*., 2010; Chen and Huang, 2010; Tlak Gajger *et al*., 2010; Traver and Fell, 2011) and indeed, in some countries it has been identified as the sole or predominant infection in *A. mellifera*. However, the prevalence of this microsporidium in other countries seems to be much lower (Giersch *et al*., 2009; Gisder *et al*., 2010; Whitaker *et al*., 2010; Yoshiyama and Kimura, 2011), which could be due to the more recent introduction of *N. ceranae* into the country in some cases (Chen and Huang, 2010), to the different climatic conditions (Giersch *et al*., 2009; Gisder *et al*., 2010) or management of the bees, differences in patogenicity of haplotypes (Williams *et al*., 2010), bees sampled or to other as yet unidentified factors.

**Table 4.** Percentage infection by *N. apis* and/or *N. ceranae* in worker bees and drones.

| | *n* | *N. ceranae* | *N. apis* | Both |
|---|---|---|---|---|
| Worker bees | | | | |
| Interior | 400 | 20.75 | 0 | 0.25 |
| Forager | 381 | 39.63 | 1.8 | 1.05 |
| Total | 781 | 29.96 | 0.9 | 0.6 |
| Drones | 85 | 38.8 | 5.9 | 20.0 |

Also, the significant differences on distribution found in this study for both *Nosema* spp. show they have different preferences for climatic conditions. In this way, *N. ceranae* seems to have predilection for hotter areas (classified as Mediterranean regions; Rivas-Martínez, 1987) and *N. apis* seems to prefer milder ones. Factors that could explain those differences could be related not only to the climatology (temperature and humidity) or altitude that have a direct effect in flora of each region, but also with the bee-keeping practices (as transhumance or the different type of hives used in Spain: Layens or Dadant), lineage of *A. mellifera* in Iberian Peninsula (North–South gradient from Lineage A to M, Cánovas *et al*., 2008) or even a mixture of all of these factors. Other studies including broader regions of the world should confirm this different pattern for *Nosema* spp. distribution.

Moreover, the differences in the seasonal patterns of these microsporidia were clearly evident in the colonies that were co-infected by both parasites in our study. Thus, *N. apis* was more frequently found in autumn (September to December) and spring (March to June), as described previously in Andalusia (Southern Spain: Orantes Bermejo and García-Fernández, 1997). However, *N. ceranae* was found irrespective of when the samples were taken, confirming that seasonal variations do not affect this microsporidium in Spain (Martín-Hernández *et al*., 2007). The seasonal pattern of *N. apis* infection was first described in a temperate climate some time ago. In 1955, the proportion of honeybees infected with *N. apis* Zander was seen to decline in summer as the old infected

 

bees die and they no longer transmit the infection to the newly emerged individuals during the flying season (Bailey, 1955). Only a few bees contract the infection in the autumn from the remaining contaminated structures in the hive, joining the winter cluster and initiating the next outbreak of the disease. Our results in Central Spain are conform to this pattern, described more than 50 years ago.

However, from our point of view the most important observation in this trial is that although *N. ceranae* was found at all times, it does not prevent *N. apis* infection or its persistence. *Nosema apis* apparently disappeared in autumn, after the outbreak, when only *N. ceranae* could be detected. However, *N. apis* was again detected some months later, in spring, confirming that *N. ceranae* infection in colonies does not impede *N. apis* from infecting the colonies. Similarly, *N. apis* did not protect colonies from *N. ceranae* infection, indicating that apparently one species of microsporidium does not exclude the other at the colony level, and there does not seem to exist a competitive interference between them. These results have been observed not only in Spain but also in other countries, such as the USA (Dr R. Cramer, pers. comm.).

When honeybees from co-infected colonies were studied at the individual level, again *N. ceranae* was the more prevalent species, both in house and forager bees, and *N. apis* was found much less frequently and was apparently more restricted to older bees (only one bee out of 400 interior bees analysed was positive for *N. apis* and in conjunction with *N. ceranae*). Foragers have been identified as the most reliable population in which to diagnose *Nosema* infection (L'Arrivée, 1963; Doull, 1965; Higes *et al*., 2008; Meana *et al*., 2010) and although co-infection of the same individual forager was observed, it was uncommon. In addition, the high level of microsporidia infection found in drones confirms the importance of this caste in the transmission of these diseases, as previously reported (Traver and Fell, 2011).

Competition occurs whenever two or more organisms use the same resources (Bush *et al*., 2001), as described between microsporidia that infect *Lymantria dispar* (Solter *et al*., 2002). In laboratory assays, neither *N. apis* nor *N. ceranae* has been shown to exert a clear competitive advantage (Forsgren and Fries, 2010) in co-infections. Our results confirm that neither of these parasites excludes the other, either at colony level or the individual level, although *N. ceranae* is the more dominant species in Spain, a country with a mostly Mediterranean climate but with a Eurosiberian climate in certain areas. Thus, it would seem that this species has some advantage over *N. apis* under field conditions, at least under the same specific climatic conditions. Indeed, when compared at the individual level, either in worker bees or drones again *N. ceranae* is clearly dominant. This would suggest that either it outcompetes *N. apis* in some way or that warm conditions are better for its development, although more research should confirm this point. This phenomenon may reflect interactive niche segregation, whereby the niche of *N. apis* may become diminished by the presence of *N. ceranae*. However, this statement is not fully supported by our work since *N. apis* is still present in Spain in a level similar to historical references and no replacement of it was observed. Future studies will be necessary to identify the different factors that may provide *N. ceranae* with an advantage to become into a higher prevalence pathogen over *N. apis*.

Finally, since most studies on *Nosema* currently use PCR to detect the microsporidia it is very important to develop a method that guarantees reliable results on which accurate conclusions can be based. In this study multiplex PCR was employed in all cases to detect the parasites, which included an internal control target (IPC) to assess the fidelity of the PCR. This IPC amplicon was observed even when PCR was performed on individual bees and it did not negatively affect the sensitivity of the assay, confirming its usefulness in improving the methods currently in use. This multiplex system will enable a diagnostic laboratory to include quality controls in the analysis of these microporidia (as recommended by Accreditation services). Therefore, the triplex PCR here developed has demonstrated a high sensitivity and specificity for *N. apis* and *N. ceranae* detection and provides to the laboratories a reliable method to ensure the diagnostic results. Recently Erler and colleagues (2011) reported low sensitivity and specificity using 218MITOC primers in quantitative PCR (Q-PCR). Changes in sensitivity should be due to the different efficiency in DNA extraction method and to the different methodology used since MITOC primers were designed for conventional PCR and not for Q-PCR. It is important to notice that a different annealing temperature was used (optimized for 61.8°C in Martín-Hernández *et al*., 2007 vs. the 58°C used in Erler *et al*., 2011).

However, regarding the specificity of 218MITOC our results strongly disagree with them since: (i) specificity of 218MITOC primers was confirmed by sequencing the PCR products and all the sequences always matched to *N. ceranae* and never to *N. apis* (Martín-Hernández *et al*., 2007); (ii) there are several sequences available in GenBank (http://www.ncbi.nlm.nih.gov) obtained by different research teams after sequencing PCR products using 218MITOC primers and all of them matched merely with *N. ceranae* (accession numbers JF276831.1; JF431547.1; JF431546.1; HM859899.1; HM859898.1 HM859897.1; HM859896.1; GU254027.2 GU205784.2; GU045468.2; GU045467.2; GU045466.2; FJ425736.1; FJ415326.1; FJ789801.1; FJ789800.1; FJ227957.1); and (iii) if 218MITOC primers would amplify *N. apis* as well, no single species infection by this microsporidium would be

detected when tested in duplex/triplex PCR, since a double band should be observed (one corresponding to 321APIS primers and the other for 218MITOC). However, this method (the three pairs of primers combined) has been extensively tested (up to now more than 15 000 samples analysed in 5 years; data not shown). During this time, many *N. apis*-infected samples have been obtained and never was observed any unspecific annealing. Moreover, the absence of sequencing of the PCR product in Erler and colleagues (2011) does not allow to exclude that the lack of specificity was in fact due to contamination during the procedure.

Furthermore, the high plasticity of COI primers (high range of annealing temperatures and sensitivity) makes them very helpful to use them as internal controls when working in PCR for the detection of different honeybee pathogens.

## Experimental procedures

### *Improvement of PCR: design of the IPC*

A search of the NCBI Taxonomy browser homepage (http://www.ncbi.nlm.nih.gov) was performed to identify published sequences of *A. mellifera ligustica*, identifying a total of 38 sequences. In order to identify possible polymorphisms between different individuals, we focused on the mitochondrial cytochrome oxidase subunit I (COI) and the NADH dehydrogenase subunit 2 (ND2) genes, because there were eight sequences for each. Thus, the eight COI sequences of 1426 bp in length (AY114452, AY114453, AY114454, AY114455, AY114456, AY114457, AY114458, AY114460) and the eight ND2 sequences 589 bp long (AY114484, AY114485, AY114486, AY114487, AY114488, AY114489, AY114490, AY114491) were compiled. Sequences from each gene were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/) to identify individual polymorphic nucleotide positions and to avoid them when designing the primers. Two consensus sequences (with variable sites named using the IUB code) were obtained for each gene.

Both sequences were checked for the formation of secondary structures using the Mfold program (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) to avoid designing primers that would produce poor PCR amplification.

Since the main aim in using these primers was to combine them with those described previously for *N. apis* and *N. ceranae* (321-APIS for *N. apis* and 218-MITOC for *N. ceranae*: Martín-Hernández *et al*., 2007), the consensus COI and ND2 sequences were assessed using Primer3 (v. 0.4.0: http://frodo.wi.mit.edu/), under the following parameters: (i) product size ranging from 100 to 200 bp; (ii) optimum primer Tm of 60°C; and (iii) optimum primer GC content of 60%. The primer pairs obtained for the consensus COI gene adjusted better to the selected parameters than those obtained in the consensus ND2 gene and thus, detection of the COI sequence with these primers was incorporated into the PCR assay.

The suitability of the primers (G/C content and melting temperatures) was checked using the IDT OligoAnalyzer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) and the Oligonucleotide Calculator programs (http://www.basic.northwestern.edu/biotools/oligocalc.html). The Tm of the primers was readjusted by adding G or GC tails to the 5′ end of both primers and thus, the final size of the expected product amplified from *A. mellifera* using the COI primers was 119 bp. Possible primer interactions between the new COI primers and the primers of duplex PCR designed previously (see above) were tested using the AutoDimer program (http://www.cstl.nist.gov/div831/strbase//AutoDimerHomepage/AutoDimerProgramHomepage.htm). Finally, species specificity was assessed by conducting a nearly exact match search with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Only significant hits with COI of *A. mellifera* subspecies were obtained.

Since the sensitivity of the primers currently used to detect *Nosema* sp. (321-APIS for *N. apis* and 218-MITOC for *N. ceranae*) and the conditions for PCR have already been defined (Martín-Hernández *et al*., 2007), we tested the COI primers at concentrations between 0.4 and 0.01 μM in triplex PCR to determine the best concentration to be used. A temperature gradient was assessed and since the primers worked very well over a large range of temperatures, we maintained the temperature of 61.8°C that was previously chosen for 321-APIS and 218-MITOC annealing.

The specificity of the COI primers was tested against different *A. mellifera* bee species (*A. m. ligustica, A. m. mellifera, A. m. carnica* and *A. m. iberiensis*), as well as with *A. cerana*, *A. florea*, *A. koschevnikovi* and some *Bombus* species (*Bombus morio*, *Bombus belicosus*, *Bombus atratus*, *Bombus tucumanus*, *Bombus dahibomii*, *Bombus opifex*).

To verify that the resulting amplicons corresponded to the expected sequences, three PCR products were purified with Qiaquick PCR Purification Kit (No. 28104 Qiagen) and fully sequenced in both directions (3730 DNA Analyzer, Applied Biosystem).

### *Sensitivity of the method for the detection of* Nosema *spp.*

To determine the sensitivity of the Extraction method and PCR used, Percoll® purified spores of *N. apis* or *N. ceranae* were counted under a contrast phase microscope and duplicate solutions of $10^6$ *N. apis* or *N. ceranae* spores were prepared in 150 μl of $H_2O$ (PCRq: equivalent $6 \times 10^6$ spores $ml^{-1}$), designated as *N. apis* purified 1 or 2 and *N. ceranae* purified 1 or 2. The same procedure was also followed using bee samples at the same concentration of the spores indicated above ($10^6$ spores of *N. apis* or *N. ceranae* in 150 μl of $H_2O$), designated as *N. apis* macerate 1 or 2 and *N. ceranae* macerate 1 or 2. These initial master solutions were diluted in $H_2O$ (PCRq) to obtain different spore concentrations (the lowest spore number tested was 2.5 spores per 150 μl). Two different replicates were made for each concentration of spores, either purified or macerates, and 150 μl of each spore solution was introduced into a 96-well plate (Qiagen) along with glass beads (2 mm of diameter, Sigma).

The plates were shaken for 6 min at 30 Hz before 30 μl of ATL buffer (Qiagen 19076) containing 20 μl of Proteinase K (Qiagen 19131) was added to each well, and the plates were incubated overnight at 56°C. Subsequently, DNA was

extracted using the BS96 DNA Tissue extraction protocol in a BioSprint apparatus (Qiagen) and the plates were frozen at –20°C until use.

The sensitivity of the extraction method and PCR was tested using DNA extracted from just one *Nosema* species and using DNA templates from both *Nosema* species at the same reaction, but always including the three sets of primers in the final PCR master mix.

### *PCR conditions*

Reactions (50 μl) were carried out in a Mastercycler® ep gradient S apparatus (Eppendorf), and each contained 25 μl of Fast Start PCR Master (No. 04710452001 Roche Diagnostic), 0.4 μM of each *Nosema* sp. primer (321-APIS-F/R and 218-MITOC-F/R), 0.03 μM of COI-F/R primers, 0.2 mg $ml^{-1}$ BSA, 0.1% Triton X-100 and 5 μl of DNA template. The thermocycler programme was: 95°C (10 min); 35 cycles of a 30 s denaturalization at 95°C, a 30 s elongation at 61.8°C and a 45 s extension at 72°C; and a final extension step at 72°C for 7 min. Negative controls (for DNA extraction and PCR) were included in all PCR experiments and all the PCR products were analysed in a QIAxcel System (Qiagen) using a QIAxcelDNA High Resolution Kit (Qiagen, no. 929002).

### *Transverse study:* Nosema *prevalence and distribution in Spain*

Determining the prevalence of *Nosema* sp. is part of a wider survey designed to study 'depopulation syndrome' or 'colony loss phenomenon' in Spain, and it included a determination of many different pathogens and pesticides. The epidemiological unit was the apiary (one colony per apiary) and the resulting sample size was calculated in relation to the number of apiaries registered in 2003 (source: Spanish Ministry of Agriculture), with an expected prevalence of depopulation of 40%, with a precision of 10% and a confidence level of 95%. The total number of samples was distributed in proportion to the number of apiaries in each region, from which the colonies were selected randomly. This cross-sectional study was carried out between spring 2006 and autumn 2007, and it involved a total of 1938 sampled apiaries distributed as follows: 629 honeybee colonies in spring 2006; 454 in autumn 2006; 526 in spring 2007; and 329 in autumn 2007. Each apiary was geo-referred and information about the type of bioclimate belt where they were located was obtained according to the 'Map of vegetation series of Spain' established by Rivas-Martínez (1987). This author described and cartographically represented different bioclimatic belts in the phytogeographical regions of the Iberian Peninsula according to climatic and altitudinal variables and vegetation attributes of the territory. In this way, the Mediterranean region shows five belts: thermo-, meso-, supra-, oro- and cryoromediterranean (from lower to higher altitude) and the Eurosiberian region shows four belts: colline, montane, subalpine and alpine.

The distribution of *Nosema* sp. was related to the agroclimatic information obtained and treated with Geographical Information Systems (GIS, v. 9.0). Pathogens distribution and proportions found were compared through Pearson $\chi^2$.

All samples were sent by the bee-keepers or veterinary services in charge of the apiaries of each bee-keeping association to the Bee Pathology Laboratory (Centro Apícola Regional, CAR). On reception, the bee samples were stored at –20°C prior to analysis.

From each sample, the abdomens of 100 bees were removed manually with a forceps and they were macerated for 2 min at high speed in 25 ml $H_2O$ (PCRq) in a Stomacher® 80 Biomaster (Seward) using filter bags (BA6040/STR, Seward). This macerate was recovered in a tube, while a further 15 ml of $H_2O$ (PCRq) was added to the stomacher bag and the residue was macerated again for the same time and speed. The solutions obtained with the bee macerates were centrifuged at 514 *g* for 10 min, the supernatant was discarded and the sediment was used for DNA extraction as described above.

### *Longitudinal study: season influence*

In order to study the natural dynamics of *Nosema* sp. infection in field conditions, 30 honeybee colonies (*A. mellifera iberiensis*) from two experimental apiaries (2 km apart) naturally infected by both *N. apis* and *N. ceranae* were selected. In Apiary 1 there were nine colonies studied from October 2006 to December 2007. In Apiary 2 there were 21 colonies studied from September 2007 to December 2008. A sample of 30 foragers was collected after closing the hive entrance (Meana *et al*., 2010) each month (except for January, March and October 2008) and stored at –20°C prior to later processing. The *Nosema* species present were determined as indicated above.

### *Individual level: prevalence of* Nosema *sp. in the hive*

*Worker bees.* To determine the prevalence of each *Nosema* species within the colonies, and the differences between young and old worker bees, samples from four colonies positive for both microsporidia were used. The samples were collected on four occasions, at month intervals. Both house bees (brushed off from no-brood combs) and forager bees (bees arriving at the colony after closing hive entrance) were collected separately from each of the four colonies, and once in the laboratory, the samples were frozen (–20°C) until the bees died. To obtain individual data, the abdomens of house and forager bees ($n = 20$ per colony, type of bees and sampling) were analysed individually by putting a single abdomen in a well of a 96-well plate with glass beads and 300 μl of water (PCRq). DNA extraction and PCR were performed as described for worker bees.

*Drones.* A total of 85 drone bees were collected from a drone-breeding colony known to have been infected by *N. ceranae* and *N. apis* since September 2007. In order to determine the prevalence of the Microsporidia in this bee caste, the abdomen of each bee was analysed individually as described for worker bees.

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# Artículo 2

## La creciente prevalencia de *Nosema ceranae* en España, un problema emergente de la última década

Cristina Botías, Raquel Martín-Hernández, Encarna Garrido-Bailón, Amelia González-Porto, Amparo Martínez-Salvador, Pilar De la Rúa, Aránzazu Meana y Mariano Higes.

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*Las microsporidiosis causadas por la infección de* N. apis *y/o* N. ceranae *se han convertido en unas de las patologías más comúnmente encontradas en las abejas melíferas. La miel almacenada en las colmenas puede estar contaminada con esporas de ambos microsporidios, siendo además una matriz adecuada para el estudio de la prevalencia de otros patógenos esporulados de las abejas. Las colecciones de mieles del Centro Apícola Regional (CAR) recibidas en distintos años desde 1988 hasta el 2009 se analizaron por PCR con el fin de identificar el momento de aparición en el tiempo de* N. ceranae *en las mismas. Para ello se analizaron un total de 240 muestras de miel que se encontraban congeladas y almacenadas, comparando los resultados obtenidos en mieles con las prevalencias de estos microsporidios en muestras de abejas obreras de diferentes años y orígenes geográficos. Además se llevó a cabo el análisis de la posible presencia de Nosema spp. en la miel almacenada dentro de colonias experimentales infectadas de manera natural por estos microsporidios, mostrando que la probabilidad de encontrar esporas de los mismos en las colonias infectadas es relativamente alta. La miel más antigua en la que se encontró esporas de* N. ceranae *se correspondió con una muestra recolectada en el año 2000.*

*En años posteriores, el número de muestras que contenían* N. ceranae *tendió a incrementarse, coincidiendo con el aumento en la prevalencia de Nosema spp. en muestras de abejas adultas procedentes de colonias. Las evidencias sobre la presencia de* N. ceranae *en nuestro país desde al menos el año 2000 podrían corroborar la hipótesis sobre el largo periodo de incubación desarrollado en las colonias afectadas por nosemosis tipo C, ya que no fue hasta el año 2004 cuando se comenzó a observar una pérdida generalizada de colonias en España. De otro modo, este fenómeno podría estar relacionado con algún factor epidemiológico desconocido.*

*La prevalencia actual de la nosemosis, principalmente debida a* N. ceranae*, ha alcanzado en España niveles de epizootia, tal y como confirman los análisis de muestras de abejas obreras y de miel comercial.*

***PRESENTACIÓN EN CONGRESO***

*Botías C, Martín-Hernández R, Garrido-Bailón E, González-Porto AV, Martínez-Salvador A, De la Rúa P, Meana A, Higes M. The increasing prevalence of nosemosis of honey bees due to* Nosema ceranae *(Microsporidia) in Spain, an emerging problem for the last decade. 42º Congreso APIMONDIA, 21-25 de Septiembre 2012, Buenos Aires (Argentina).*

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# The growing prevalence of *Nosema ceranae* in honey bees in Spain, an emerging problem for the last decade

Cristina Botías [a], Raquel Martín-Hernández [a,b], Encarna Garrido-Bailón [a], Amelia González-Porto [a], Amparo Martínez-Salvador [c], Pilar De La Rúa [d], Aránzazu Meana [e], Mariano Higes [a,*]

[a] Centro Apícola Regional (CAR), Dirección General de la Producción Agropecuaria, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, 19180 Marchamalo, Spain
[b] Instituto de Recursos Humanos Para la Ciencia y la Tecnología (INCRECYT), Fundación Parque Científico y Tecnológico de Albacete (ESF), Spain
[c] Departamento de Epidemiología, TRAGSATEC, Madrid, Spain
[d] Departamento de Zoología y Antropología Física, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain
[e] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain

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ABSTRACT

Microsporidiosis caused by infection with *Nosema apis* or *Nosema ceranae* has become one of the most widespread diseases of honey bees and can cause important economic losses for beekeepers. Honey can be contaminated by spores of both species and it has been reported as a suitable matrix to study the field prevalence of other honey bee sporulated pathogens. Historical honey sample collections from the CAR laboratory (Centro Apícola Regional) were analyzed by PCR to identify the earliest instance of emergence, and to determine whether the presence of *Nosema* spp. in honey was linked to the spread of these microsporidia in honey bee apiaries. A total of 240 frozen honey samples were analyzed by PCR and the results compared with rates of *Nosema* spp. infection in worker bee samples from different years and geographical areas. The presence of *Nosema* spp. in hive-stored honey from naturally infected honey bee colonies (from an experimental apiary) was also monitored, and although collected honey bees resulted in a more suitable sample to study the presence of microsporidian parasites in the colonies, a high probability of finding *Nosema* spp. in their hive-stored honey was observed. The first honey sample in which *N. ceranae* was detected dates back to the year 2000. In subsequent years, the number of samples containing *N. ceranae* tended to increase, as did the detection of *Nosema* spp. in adult worker bees. The presence of *N. ceranae* as early as 2000, long before generalized bee depopulation and colony losses in 2004 may be consistent with a long incubation period for nosemosis type C or related with other unknown factors. The current prevalence of nosemosis, primarily due to *N. ceranae*, has reached epidemic levels in Spain as confirmed by the analysis of worker honey bees and commercial honey.



## 1. Introduction

Microsporidiosis caused by microsporidia of the genus *Nosema* is one of the most widespread disease of adult honey bees, and it is responsible for significant economic losses for beekeepers worldwide acting alone (Higes et al., 2008a, 2009; Hornitzky, 2008; Heintz et al., 2011) or in combination with other synergistic factors (Alaux et al., 2010; Bromenshenk et al., 2010; Bacandritsos et al., 2010; Vidau et al., 2011). Two species of microsporidia infect *Apis mellifera*, namely *Nosema apis* and *Nosema ceranae*. *N. apis* was first described over a 100 years ago, and its effects on individual bees and whole colonies have been widely documented (revised by Neveu-Lemaire (1938); Bailey and Ball (1991); OIE (2008)). By contrast, *N. ceranae* is a microsporidium of the Asian bee *Apis cerana* (Fries et al., 1996) that was recently shown to parasitize *A. mellifera* (Higes et al., 2006; Huang et al., 2007) and is now detected worldwide as one of the predominant microsporidia infecting honey bees (Martín-Hernández et al., 2007; Chaimanee et al., 2010; Chen and Huang, 2010; Higes et al., 2010a; Suwannapong et al., 2010; Tlak Gajger et al., 2010; Stevanovic et al., 2011), even though it seems that *N. apis* may be more prevalent in colder climates (reviewed by Fries (2010); Gisder et al. (2010a)). Both microsporidia are obligate intracellular parasites that infect epithelial cells of the honey bee ventriculum after ingestion of spores, although they cause two distinct illnesses with different epidemiological patterns, clinical signs, and consequences in terms of honey bee and colony viability (Chen and Huang, 2010; Gisder et al., 2010b; reviewed in Higes et al., 2010a; Bourgeois et al., 2011). Indeed, there are data suggesting that *N. ceranae* is more adaptable and less host specific than *N. apis* (Plischuk et al., 2009; Martín-Hernández et al., 2009; Chaimanee et al., 2010; Suwannapong et al., 2010; Chen and Huang, 2010; Higes et al., 2010b). Hence, it has been recently proposed to distinguish between the nosemosis caused by *N. apis* (nosemosis type A) and the disease caused by

* Corresponding author. Tel.: +34 949 250026; fax: +34 949 250176.
*E-mail address:* mhiges@jccm.es (M. Higes).

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*N. ceranae* by using the term nosemosis type C to define the infections caused by the latter agent (Higes et al., 2010a).

Several fomites have been proposed as being responsible for the spread of *Nosema* spp. spores in beekeeping, including corbicular pollen (Higes et al., 2008b), wax (Malone and Gatehouse, 1998), royal jelly (Cox-Foster et al., 2007), beekeeping material (Van der Zee, 2010), and regurgitated pellets of *Merops apiaster* (Higes et al., 2008c; Valera et al., 2011). Honey, the main product of the hive, can also be contaminated by *N. apis* or *N. ceranae* spores (Giersch et al., 2009) and in the past, it has been used as a matrix to detect sanitary problems, such as *Paenibacillus larvae* infection in bee colonies (Antúnez et al., 2004; de Graaf et al., 2001; Ritter, 2003). Hence, honey may serve as a medium to study the field prevalence of other honey bee sporulated pathogens when honey bee samples are not available.

On the basis of historical data, it has been assumed that *N. ceranae* arrived in Europe during the last decade of 20th century (Martín-Hernández et al., 2007; Klee et al., 2007), although this remains to be confirmed (Fries, 2010). Indeed, old honey bee samples from the collections of honey bee laboratories are not always available or suitable for molecular analysis. However, many laboratories store honey samples to determine their floral origin and often, these samples have been kept for long periods of time in conditions that preclude external contamination by spores or DNA of pathogens. The aim of our study was to identify the earliest presence of *N. ceranae* in honey samples from professional apiaries in Spain, and to determine whether its presence in honey is correlated with the increase in the prevalence of this microsporidium observed over the years in honey bee apiaries. Finally, the likelihood of detecting *Nosema* spp. in hive-stored honey from natural infected honey bee colonies in field conditions was also examined.

**Table 1**
Percentage of historical honey samples and honey bee samples positive to *Nosema* spp. Honey samples were analyzed by PCR for the detection of *N. apis* and *N. ceranae*, and honey bee samples were examined by PCM 400× to detect *Nosema* spores presence.

| Years | Honey samples (PCR analysis) | | | Honey bee samples (PCM 400×) | |
|---|---|---|---|---|---|
| | *N* | Positive honey samples (%) | | *N* | Positive honey bee samples (%) |
| | | *N. ceranae* | *N apis* | | *Nosema* spp. |
| 1988 | 1 | 0 | 0 | | |
| 1991 | 4 | 0 | 0 | | |
| 1992 | 4 | 0 | 0 | | |
| 1993 | 20 | 0 | 0 | | |
| 1994 | 16 | 0 | 0 | | |
| 1995 | 11 | 0 | 0 | | |
| 1996 | 5 | 0 | 0 | | |
| 1997 | 7 | 0 | 14.3 | | |
| 1998 | 5* | 0 | 0 | | |
| 1999 | 14* | 0 | 0 | 154** | 13 |
| 2000 | 16 | 6.3 | 0 | 124** | 10 |
| 2001 | 14* | 6.7 | 0 | 146** | 25 |
| 2002 | 10 | 0 | 0 | 443** | 23 |
| 2003 | 1 | 100 | 0 | 484** | 43 |
| 2004 | 12 | 16.7 | 0 | 3002** | 89 |
| 2005 | 17 | 17.6 | 0 | 1423** | 95 |
| 2006 | 16 | 18.8 | 0 | 1778 | 85 |
| 2007 | 31 | 32.3 | 0 | 1912 | 84 |
| 2008 | 18 | 22.2 | 0 | 1238 | 87 |
| 2009 | 15 | 33.3 | 0 | 787 | 85 |
| TOTAL | 240 | 12.7 | 0.4 | 11491 | 80.6 |

* One of the samples in each of these years did not show evidences of DNA integrity (no COI gene of *A. mellifera* amplified). These three samples were not taken into account when the prevalence was calculated.
** Data extracted from Martín-Hernández et al. (2007).

## 2. Materials and methods

### 2.1. Historical honey samples

The honey samples used in this study were sent to the Hive-Products Laboratory of Centro Apícola Regional (CAR) by Spanish beekeepers from 1988 to 2009. On receipt, the botanical origin was determined by palynological analysis (as described in Higes et al. (2008a)) and organoleptic or physicochemical features were detailed. After analysis, these samples were stored in the origin collection jar (limiting possible contamination) and they were stored at −18 °C until the present study. No contamination of samples with *Nosema* spp. spores or DNA could occur at any time during storage, as adult worker bees are never studied in this laboratory. Thus, the results are representative of the honey at the time of harvesting from the colony.

Samples from peninsular Spanish territories were selected randomly for analysis in this study (Table 1). After thawing at room temperature, the samples were incubated for 30 min at 40 °C and homogenized by stirring. A 25–50 ml aliquot of honey was obtained from each sample and PCR analysis of *Nosema* spp. was performed using a modification of the method described by Giersch et al. (2009). Briefly, the 25 ml aliquot of each honey sample was mixed with an equal volume of phosphate buffered saline (PBS, 0.1 M) and centrifuged for 45 min at 3000*g*. The supernatant was discarded and the pellet was washed with 10 ml PBS. This solution was centrifuged for 15 min at 15,000*g* and the final pellet re-suspended in 0.5 ml PBS.

### 2.2. Prevalence of Nosema in professional apiaries

To determine the prevalence of nosemosis in bee samples for later comparison with the prevalence of *Nosema* presence in commercial honey, we used data from adult worker bee samples sent to the CAR laboratory by beekeepers or veterinary services associated with apiaries in various regions of Spain that experienced pathological problems or colony losses. Specifically, we used records of *Nosema* prevalence from 1999 to 2005 reported in a previous study (Martín-Hernández et al., 2007), and results obtained between 2006 and 2009 (7138 worker honey bee samples analyzed for a national survey). All analyses were made following modified OIE Methods (2004). Briefly, 20–100 worker bees (according to the number of bees available per sample) were macerated in distilled water, centrifuged for 6 min at 800*g*, and the pellet was re-suspended in 1 ml of distilled water. Fresh preparations from the resulting homogenates were analyzed by Phase Contrast Microscopy (PCM) at 400× magnification.

To determine which *Nosema* species was more prevalent and whether any changes in prevalence occurred over a 5 year period, we analyzed adult bee samples from two random surveys conducted in three Spanish regions in spring 2002 and 2007: Catalonia, Valencia (both in the Mediterranean area), and Andalusia (Southern Spain). Together, these regions contained representatives of 40% (year 2002) and 44% (year 2007) of the total number of Spanish honey bee colonies (data from the Spanish "Ministerio de Medio Ambiente, Rural y Marino"). Bees from the 2002 survey were collected for genetic studies by Dr. De la Rúas team and they were stored in absolute ethanol in the Animal Biology Area of the Veterinary Faculty in Murcia. Bees from the 2007 survey were collected as part of a national survey that included other Spanish territories (Garrido-Bailón et al., 2008) and they were maintained frozen (−20 °C) at the CAR laboratory until they were analyzed for *Nosema* spp. by PCR. In these studies, the abdomens of worker bees (30 bees per sample in 2002 survey; 100 bees per sample in 2007 survey) were removed manually using sterile forceps and every composite sample was macerated in 25 ml $H_2O$ PCR grade (PCRq) for 2 min at high speed in a

**Table 2**
Prevalence and confidence interval (CI, 95%) of *Nosema* in bees in three different regions of Spain in 2002 and 2007 (*N* = number of samples; *n* = number of positive samples).

| Region | Year | *N* | *N. ceranae* | | | | *N. apis* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *n* | % | CI (95%) | Pearson $\chi^{2*}$ (*P*-value) | *n* | % | CI (95%) | Pearson $\chi^{2*}$ (*P*-value) |
| Andalusia | 2002 | 80 | 23 | 28.8 | 18.2–39.29 | 0.000** | 8 | 10 | 2.8–17.2 | 0.780 |
| | 2007 | 80 | 52 | 65 | 53.1–75.3 | | 6 | 7.5 | 1.1–13.9 | |
| Catalonia | 2002 | 30 | 2 | 6.7 | 0.8–22.1 | 0.470 | 2 | 6.7 | 0.8–22.1 | 0.947 |
| | 2007 | 9 | 2 | 22.2 | 2.8–60.0 | | 0 | 0 | 0.0–33.6 | |
| Valencia | 2002 | 34 | 9 | 26.5 | 10.2–42.8 | 0.248 | 8 | 23.5 | 7.8–39.3 | 0.533 |
| | 2007 | 17 | 8 | 47.1 | 22.9–72.2 | | 2 | 11.8 | 1.5–36.4 | |
| TOTAL | 2002 | 144 | 34 | 23.6 | 16.3–30.9 | 0.000** | 18 | 12.5 | 6.8–18.2 | 0.290 |
| | 2007 | 106 | 62 | 58.5 | 48.1–67.8 | | 8 | 7.5 | 2.1–13.1 | |

* Pearson or Yates correction when necessary.
** $P < 0.01$, statistically significant differences found.

Stomacher® 80 Biomaster (Seward, West Sussex, UK) using strainer bags (BA6040/STR, Seward). The macerate was recovered in a tube, and 15 ml of $H_2O$ (PCRq) was added to the strainer bag and the maceration repeated under the same conditions. Honey bee macerates were centrifuged as described previously, the supernatant was discarded and DNA was extracted from the sediment.

Pearson's $\chi^2$ test (with Yates correction when needed) was used to compare the prevalence of *N. apis* and *N. ceranae* between 2002 and 2007. A Pearson's correlation coefficient was calculated between the data of prevalence obtained in this study and the results of prevalence obtained from the historical honey samples in order to detect statistically significant linear dependence ($P < 0.01$) between both groups of variables.

### 2.3. Honey and bee samples from Nosema positive dead colonies

To ascertain the link between the presence of *Nosema* spp. in hive-stored honey and natural infected honey bee colonies in field conditions, honey samples from 15 naturally infected experimental colonies were collected aseptically from the brood chamber when the colonies collapsed (CAR apiaries). These colonies had been monitored since 2007 and adult bees had been periodically sampled until the final collapse to assess *Nosema* infection by PCR analysis of foragers. Both honey and bee samples were processed as described above.

### 2.4. PCR analysis

DNA was extracted from the re-suspended bee or honey pellets. Concisely, 150 μl of sediment was placed in a 96-well plate (Qiagen, Hilden, DE) containing glass beads (2 mm diameter, Sigma) and at least one blank well containing water as a negative control for the extraction was included per 20 bee or honey samples. The plates were shaken in a TissueLyser machine (Qiagen) for 6 min at 30 Hz, after which 30 μl of ATL buffer (Qiagen 19076) and 20 μl of Proteinase K (Qiagen 19131) was added to each well, and the plates were incubated overnight at 56 °C. DNA was then extracted using the BS96 DNA tissue extraction protocol in a BioSprint (Qiagen). Finally, the plates containing DNA were frozen at −20 °C until use.

The DNA extracted was analyzed by PCR to determine the *Nosema* species present using the specific 218MITOC F/R and 321APIS F/R primers described previously (Martín-Hernández et al., 2007). PCR conditions were as follows: in 25 μl containing 12.5 μl of Fast Start PCR Master (No. 04710452001 Roche Diagnostic, Basel, CH), 0.4 μM of each pair of primers, 0.2 mg/ml BSA, 0.1% Triton X-100 and 2.5 μl of DNA template. Thermocycler program: 95 °C (10 min); 35 cycles of 95 °C (30 s), 61.8 °C (30 s), and 72 °C (45 s); final extension 72 °C (7 min). Sensitivity levels of this technique are 2.5 spores of *N. ceranae* or 25 spores of *N. apis* in 150 μl of bee macerate (CAR laboratory, unpublished data).

To test the level of preservation of DNA in the long-term stored honey samples and to ensure that negative results were not due to a degradation of DNA, cytochrome c-oxidase (COI) gene of *A. mellifera* was used as a positive control of the integrity of DNA contained in all the studied honey samples. The COI primers sequences used for positive control were COI-F (GGGTCCAAGACCAGGAACTGGAT) and COI-R (GCGCGGAAATTCCTGATATATGAAGAGAAAA) (Martín-Hernández et al., unpublished data). The PCR and cycling conditions were the same as described above, but using 0.2 μM of COI-F/R primers.

Each PCR product was analyzed in QIAxcel System (Qiagen), using QIAxcel DNA Resolution Kit (QIAgen, No. 929002).

Negative and positive controls were processed in parallel during the extraction and PCR phases of analysis in order to detect possible contamination and assess the reliability of sample processing.

## 3. Results

A total of 240 frozen honey samples (26.7% of the total honey samples stored at the laboratory with accompanying geographical data) from 26 different Spanish territories were analyzed for the presence of *N. ceranae* or *N. apis*. The most prevalent microsporidium detected was *N. ceranae*, which was found in analyzed honey samples since 2000 until 2009, with the exception of 2002 (Table 1). The pollen spectrum of each honey confirmed the botanical origin declared by beekeeper in all cases and no imported honey was analyzed.

The analysis of the COI gene of *A. mellifera* in the honey samples tested positive for *A. mellifera* presence in all the studied samples except for one sample from the following years: 1998, 1999, and 2001, that tested also negative to *Nosema* spp. These samples were excluded from the analysis of prevalence, so the total of 240 honey samples was reduced to 237.

None of the honey samples collected prior to 1997 tested positive for either microsporidia. In this year, *N. apis* was detected in a sample from Central Spain (Ciudad Real). The oldest honey sample to test positive for *N. ceranae* in this study was obtained in 2000 in a sample from the central area of Spain (Cuenca). The number of samples positive for *N. ceranae* tended to increase in subsequent years, as did the detection of *Nosema*-positive worker bee samples from apiaries with various pathological problems or higher autumn–winter colony losses (Table 1). A statistically significant correlation ($r = 0.835$; $P = 0.003$) between the prevalence of *Nosema* spp. in worker bee samples sent by beekeepers and the detection of microsporidia in honey was obtained (honey data from 2003 was removed for the correlation study because it was just only

one data positive to *N. ceranae* that meant 100%, see Table 1), showing both a trend of increase after year 2000.

The prevalence of *Nosema* spp. in the surveys carried out in three Spanish regions was 30.2% in 2002 and 47.2% in 2007. Colonies sampled represented a high proportion of Spanish honey bee colonies. This trend of increased prevalence was observed in all three areas and was related to a raise in the presence of *N. ceranae* (Table 2), although this effect was only statistically significant in Andalusia, where the greatest number of samples was analyzed, and also when the data from the three regions were combined (Pearson $\chi^2$, $P < 0.01$). By contrast, there appeared to be a slight reduction in the prevalence of *N. apis* in all these regions, but this effect was not significant for any year or region studied (Pearson $\chi^2$, $P > 0.05$; Table 2).

Of the experimental colonies naturally infected in field conditions, all 15 collapsed colonies tested positive for *N. ceranae* in the month prior to collapse (as determined by PCR analysis of foragers). Moreover, in two colonies co-infection with *N. apis* was evident (13%). When colonies collapsed (each one at a different date), *N. ceranae* was detected in 13 of the 15 hive-stored honey samples analyzed (87%), while the remaining two tested negative for *Nosema* spp. (13%). All these colonies stayed infected by *Nosema* spp. during 15 months in average (range 9–33 months).

## 4. Discussion

We demonstrate here for the first time that frozen honey samples can be used to identify the emergence of nosemosis due to *N. ceranae* in honey bee colonies in Spain. While extreme caution is required to avoid cross contamination when using molecular techniques to analyse honey samples (Giersch et al., 2009), these risks were addressed by processing negative controls along with the samples at different phases of extraction and PCR to ensure that the results of the analysis were not misleading. Thus, the results obtained provide an accurate picture of the status of the honey bee colonies in the year of honey production, with no risk of more recent contamination of the samples. Besides, the use of the COI gene of *A. mellifera* as a positive control to test DNA integrity in long-stored honey samples enabled verification of the high level of preservation of DNA even in the oldest honeys (e.g. 1988, 1991, and 1992).

Honey was found as a suitable indicator of *N. ceranae* prevalence in bee colonies under field conditions as suggested by the correlation found between the increased presence of this microsporidium in honey samples from the year 2005 onward and in randomly collected worker honey bee samples from professional apiaries during the same period.

In a previous study carried out in Australia, *N. apis* was found to be more prevalent in honey than *N. ceranae*, which was only detected in honey samples collected from regions with the highest prevalence (Giersch et al., 2009). Our results are consistent with this observation and reveal a correlation between the percentage of honey samples containing the microsporidium *N. ceranae* and the prevalence of nosemosis in honey bee colonies in Spain, as mentioned previously. In general, the epidemiological pattern of *N. ceranae* infection differed from that described for *N. apis* in temperate areas of Europe (revised by Higes et al. (2010a)). Specifically, colonies affected by *N. apis* generally display mild infection during summer, a small peak in autumn and the usually slow increase in infection during winter (Bailey, 1955; Hornitzky, 2005; Langridge, 1961) whereas *N. ceranae* can be detected in samples throughout the year even in summer months with an evident lack of seasonality under certain climatic conditions (Martín-Hernández et al., 2007; Tapaszti et al., 2009; Giersch et al., 2009) causing the now called nosemosis type C, very prevalent in our country (Higes et al., 2010c). On the other hand, several studies report that *N. apis* prevalence in our country is lower and has not increased in the last decades (Gómez Pajuelo and Fernández Arroyo, 1979; Orantes Bermejo and García-Fernández, 1997; Garrido-Bailón et al., 2008). Hence, the detection of *N. ceranae* in hive-stored honey samples from professional apiaries in Spain is more likely than detection of its congener *N. apis*.

The analysis of honey samples proved a useful means of studying the emergence of *N. ceranae* in professional apiaries in continental Spain, demonstrating the existence of this microsporidium since at least 2000. The detection of *N. ceranae* presence at this time, 4 years prior to the emergence of symptoms related to depopulation and colony losses in Spain (Higes et al., 2005) could indicate a slow spread of this microsporidian parasite following its first appearance and may reveal that signs of nosemosis type C were not evident until the pathogen was well established. Otherwise, this fact might be reflective of a long incubation period for this disease (Higes et al., 2008a). Moreover, several studies have reported the presence of *N. ceranae* in honey bee colonies in the absence of any clinical sign of nosemosis or colony declines (Genersch et al., 2010; Vejsnæs et al., 2010; Stevanovic et al., 2011), so unknown factors may be involved in triggering an acute deadly infection by this microsporidium under certain circumstances.

Data obtained from clinical honey bee samples from 1999–2009 confirmed the stable high prevalence of *Nosema* spp. in samples from sick or collapsed colonies over the last 5 years. Previous studies also indicated that the actual prevalence in professional apiaries in our country was over 40% in 2006 and 2007 (Garrido-Bailón et al., 2008), in agreement with our findings. This prevalence appears to be primarily due to an increase in *N. ceranae* rather than *N. apis*, as confirmed in bee samples collected in surveys conducted in 2002 and 2007 in three regions of Spain. A high prevalence of *N. ceranae* both in terms of time and location was observed in 2007. It is also noteworthy that sample size was higher in the case of 2007 survey (100 bees per sample) comparing with 2002 survey, factor which may also have contributed to such differences, but since 2002 survey sample size (30 bees per sample) was not too limited and bigger than the recommended by OIE standards (OIE, 2008) a remarkable influence of this factor is discarded. Besides, the lower limit of detection of our technique for *N. apis* in comparison to the presented for *N. ceranae* might have also influenced the dissimilarities observed. In this case, as the sensitivity of the technique is still very high (25 spores detected in 150 μl of bee macerate), despite being lower than the presented for the *N. ceranae* detection, a relevant impact of this factor over the results is also discarded.

The prevalence of *N. apis* over time and between areas revealed a lower prevalence and higher variability. The trend of increase in the prevalence of *N. ceranae* in the last decade in Spain is clearly supported by the raise in the detection of this microsporidium in commercial honey during this period. The prevalence of *Nosema* in these samples from asymptomatic colonies (Higes et al., 2008a) may probably be more closely related with the regional survey in which samples were randomly selected, than the data obtained from clinical samples sent to the CAR lab, which are frequently associated with bee/or colony disease.

It has been suggested that a prevalence of insect microsporidia of over 10% is indicative of an epizootic condition (Brooks, 1979). As such, our findings are suggestive of epidemic levels of *N. ceranae* infection in Spanish territories over the last decade, which have resulted in significant bee and colony losses (Higes et al., 2005, 2006, 2008a, 2009, 2010c). The absence of *N. apis* in honey after 1997 cannot be explained solely by its substitution with *N. ceranae* in infected colonies. As verified by analysis of hive-stored honey from collapsed colonies, *N. apis* may be present in forager bees without being detected in the honey from the same colony. This suggests that the presence of *N. apis* in honey is much lower than that in

bee samples, and probably the parasitic load presented by this microsporidium in the individual colonies may be lesser than the achieved by *N. ceranae* (Martín-Hernández et al., unpublished data). Similarly, the stronger prevalence of *N. ceranae* detected in bee samples may imply an increased likelihood of detecting spores in the honey samples as mentioned above.

The high percentage of *N. ceranae* positive honey samples from the collapsed experimental colonies suggests that there is a high probability of detecting this microsporidium in the hive-stored honey of a honey bee colony that has been naturally infected. Indeed, the presence of this microsporidium was detected in the hive-stored honey samples in 87% of the experimental colonies, which had been infected with *N. ceranae* for more than 9 months. This result may indicate that, although the probability of finding this microsporidium in the hive-stored honey of an infected colony is highly probable, honey bee samples are the most suitable medium to determine its presence in a colony.

Unknown factors may play a role in the storage and presence of *Nosema* spp. in the hive-stored honey, since 13% of the infected colonies did not show *Nosema* presence in the corresponding hive-stored honey. Also, the stochastic effect associated to the sampling method may have an influence in the chance of detecting the microsporidian presence. The prevalence of *Nosema* spores in honey bee samples from the present study has shown to be much higher than in hive-stored honey samples in the same period. This fact may suggest that a high percentage of historical honey samples analyzed came from non-infected colonies, but further research is needed to clarify this matter.

The current prevalence of microsporidiosis caused by *N. ceranae* detected in clinical samples and by survey indicates epidemic levels of this disease in our country, mainly due to infection with *N. ceranae,* as confirmed by the analysis of honey samples. Classical symptoms of *N apis* infection (nosemosis type A), such as crawling bees or the presence of faces in the frames and the interior of the hive, were not reported by beekeepers or observed in dead colonies for several months after *Nosema* detection (Higes et al., 2008a, 2009; Borneck et al., 2010). *N. apis* infection is characterized by the presence of spores in the gut, with transmission from older infected bees to younger ones occurring via faces or adults crushed during beekeeping (OIE, 2008). By contrast, transmission of *N. ceranae* is poorly understood. Corbicular pollen (Higes et al., 2008b) and beekeeping material (Van der Zee, 2010) have been identified as modes of transmission of *N. ceranae*, as for other pathogens. Previous reports have established a relationship between honey contaminated with *P. larvae* spores and the presence of bee colonies with American foulbrood (AFB) clinical symptoms (Antúnez et al., 2004; de Graaf et al., 2001). Moreover, it has been suggested that the early detection of AFB in a colony is possible by examination of extracted honey and visualization of spores of this bacterium (Ritter, 2003). However, given that *N. apis* spores lose viability after 1 month in honey (Malone et al., 2001), infectivity tests will be required to confirm the role of honey as a fomite of *N. ceranae*.

It was recently suggested that *N. ceranae* has no competitive advantage over *N. apis* in mixed infections when spores are introduced simultaneously in laboratory experiments (Forsgren and Fries, 2010). However, the worldwide prevalence of *N. ceranae* is considerably higher than that of *N. apis* (Chen et al., 2009; Chaimanee et al., 2010; Stevanovic et al., 2011; Tlak Gajger et al., 2010; Valera et al., 2011; Yoshiyama and Kimura, 2011) with some exceptions (reviewed by Fries (2010) and Gisder et al. (2010a)), suggesting that the former outcompetes *N. apis* under natural conditions, or at least under some circumstances that remain unknown. Replacement of *N. apis* by *N. ceranae* has also been suggested (Klee et al., 2007; Chen et al., 2008; Yoshiyama and Kimura, 2011). Although we detected a reduction in the prevalence of *N. apis* in 2007 when compared to 2002, this difference was not significant and further studies will be necessary to determine whether such a proposed replacement does in fact occur.

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# Artículo 3

## Más pruebas sobre el origen oriental de *Nosema ceranae* (Microsporidia: Nosematidae)

Cristina Botías, Denis L. Anderson, Aránzazu Meana, Encarna Garrido-Bailón, Raquel Martín-Hernández y Mariano Higes

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*A pesar de que* Nosema ceranae *fue aislado por primera vez en la abeja asiática (*Apis cerana*) y posteriormente detectado como un parásito del ventrículo de las abejas melíferas (*Apis mellifera*) ampliamente distribuido, sus orígenes y hospedador primario siguen sin ser haber sido determinados. En este estudio se evaluó la posibilidad de un origen asiático para este parásito, principalmente mediante pruebas sobre su dispersión incipiente en hospedadores fuera de Asia. Para ello, se hizo un análisis de la presencia de* N. ceranae *en poblaciones de* A. cerana *y* A. mellifera *en regiones aisladas de las Islas Salomón (región Pacífica). Muchas de estas islas fueron colonizadas por poblaciones de A. mellifera procedentes de Australia y Nueva Zelanda en una época en la que* N. ceranae *no estaba presente en dichos países. Por otro lado, en años recientes, dichas islas fueron invadidas de manera incontrolada por poblaciones de* A. cerana *procedentes de Papúa Nueva Guinea, las cuales nunca tuvieron contacto con poblaciones de A. mellifera infectadas por* N. ceranae*. La situación particular de las Islas Salomón permitió investigar la posibilidad de una infección reciente de este microsporidio en las poblaciones de A. mellifera presentes en estas islas, la cual se habría producido como consecuencia decontacto con las poblaciones invasoras e infectadas de la especie* A. cerana*.*

*Asimismo, en este ensayo fueron evaluados y verificados los datos previos sobre la amplia distribución de* N. ceranae *en Asia, a través del estudio sobre la presencia de este microsporidio en poblaciones aisladas de abejas de las especies* A. cerana*,* A. koschevnikovi*,* A. nigrocincta *y* A. florea*.*

*En este ensayo se obtuvieron evidencias sobre la posible introducción de* N. ceranae *por parte de* A. cerana *en las poblaciones de* A. mellifera *en las Islas Salomón. Además, se confirmó la amplia distribución de este microsporidio en poblaciones de abejas asiáticas, encontrándolo por primera vez en una muestra de la especie* A. koschevnikovi *procedente de la isla de Borneo.*

*Estos hallazgos proporcionan un mayor apoyo a la hipótesis que sostiene que* N. ceranae *ha emergido recientemente de Asia para convertirse en un parásito de* A. mellifera*.*

***PRESENTACIÓN EN CONGRESO:***

*Botías C., Martín-Hernández R., Garrido-Bailón E., Higes M., Anderson D.* Nosema ceranae *is able to infect different* Apis *species. 41º Congreso APIMONDIA, 15-20 de Septiembre 2009, Montpellier (Francia)*

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# Further evidence of an oriental origin for *Nosema ceranae* (Microsporidia: Nosematidae)

Cristina Botías [a,*], Denis L. Anderson [b], Aránzazu Meana [c], Encarna Garrido-Bailón [a], Raquel Martín-Hernández [a,d], Mariano Higes [a]

[a] Laboratorio de Patología Apícola, Centro Apícola Regional, CAR, Dirección General de la Producción Agropecuaria, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, 19180 Marchamalo, Spain
[b] CSIRO Ecosystem Sciences, PO Box 1700, Canberra, ACT 2601, Australia
[c] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain
[d] Instituto de Recursos Humanos para la Ciencia y Tecnología, INCRECYT (FEDER Funds), Parque Científico de Albacete, Spain

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ABSTRACT

Although *Nosema ceranae* was first isolated from the Asian honeybee (*Apis cerana*) in Asia and then subsequently recognized as a widespread gut parasite of the Western honeybee (*Apis mellifera*), its origins and primary host are yet to be accurately established. In this study we examined the possibility of an Asian origin for the parasite by looking for evidence of its ongoing spread out of Asia. To do this, we surveyed for the presence of *N. ceranae* in *A. cerana* and *A. mellifera* on isolated islands of the Solomon Islands (Pacific region), most of which were inhabited with *A. mellifera* that had been introduced from Australia and New Zealand at a time when *N. ceranae* was not present in either country, but on which some had also recently become inhabited with invasive *A. cerana* that originated from Asia with no prior history of contact with *A. mellifera* infected with *N. ceranae*. We also sought to verify previous findings that *N. ceranae* was widespread in Asian honeybees by surveying for its presence in isolated populations of the Asian honeybees, *A. cerana*, *A. koschevnikovi*, *A. nigrocincta* and *A. florea*. We obtained evidence that *A. cerana* introduced *N. ceranae* to *A. mellifera* in the Solomon Islands and also confirmed the widespread occurrence of the parasite in Asian honeybees, even reporting it for the first time in *A. koschevnikovi* from Borneo.

Our findings provide further support for the hypothesis that *N. ceranae* has only recently emerged from Asia to become a parasite of *A. mellifera*.



## 1. Introduction

The origin and primary host of *Nosema ceranae* (Microsporidia: Nosematidae), a recently discovered and widespread obligate intracellular gut parasite of honeybees (*Apis* spp.), is yet to be accurately established (Higes et al., 2010; Huang et al., 2007). The parasite was first isolated from the Eastern honeybee (*A. cerana*) in China (Fries et al., 1996) and subsequently isolated from Western honeybees (*A. mellifera*) in many regions of the world, including Europe, Africa, Asia, the Americas and Oceania (Calderón et al., 2008; Chen and Huang, 2010; Giersch et al., 2009; Higes et al., 2006, 2009; Williams et al., 2008). Recently, it was also isolated from other *Apis* species in Asia (Chaimanee et al., 2010), and from several native bumblebees (*Bombus* spp.) in Argentina (Plischuk et al., 2009).

When *N. ceranae* was initially found in *A. mellifera* in some parts of Europe its prevalence was low (Klee et al., 2007), whereas its prevalence in those regions in now high (Bacandritsos et al., 2010; Chauzat et al., 2007; Martín-Hernández et al., 2007; Tapaszti et al., 2009; Tlak Gajger et al., 2010). Furthermore, studies during the 1990s that employed rDNA gene markers capable of detecting *N. ceranae*, failed to detect it in *A. mellifera* from eastern Australia, (South Australia, the Australian Capital Territory and New South Wales) (Rice, 1999) or New Zealand (Gatehouse et al., 1999; Malone et al., 1994), even though *N. ceranae* is now common in *A. mellifera* throughout eastern Australia, but still not in Western Australia (Cox-Foster et al., 2007; Giersch et al., 2009; Hornitzky, 2008) and was detected for the first time in New Zealand in 2010 (Ministry of Agriculture and Forestry (MAF) New Zealand, 2010). These apparent late and gradual invasions of *N. ceranae* into different *A. mellifera* populations previously shown to be infected with only *N. apis*, as well as isolations of *N. ceranae* from Asian honeybees outside of China indicating it is widespread in Asian honeybees, have led some authors to suggest that *A. mellifera* may be the primary host of *N. apis*, and that *N. ceranae* may have only recently emerged as a parasite of *A. mellifera*, possibly from Asian honeybees (Cornman et al., 2009; Fries et al., 2006; Higes et al., 2006; Klee et al., 2007).

* Corresponding author. Fax: +34 949 250176.
*E-mail addresses:* cbotias@jccm.es, cristinabotias@yahoo.es (C. Botías), Denis.Anderson@csiro.au (D.L. Anderson).

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A major problem in determining the origins and primary host of *N. ceranae* has been that identifications of *Nosema* in *A. mellifera* prior to the mid-1990s were mostly based on morphological characteristics of *Nosema* spores, of which, to the untrained eye, those of *N. apis* and *N. ceranae* appear similar. The recent application of DNA markers to identify *Nosema* spp. in *A. mellifera* improved the specificity of detection but, at the same time, also showed that *N. apis* and *N. ceranae* were both present in some *A. mellifera* populations when it was assumed that only *N. apis* was present (Chen et al., 2008; Paxton et al., 2007). The ability of *N. ceranae* to utilize more than one host (Chaimanee et al., 2010; Plischuk et al., 2009; Suwannapong et al., 2010a,b) only adds to the difficulty of determining the origins and primary host of this parasite.

If *N. ceranae* originated in Asia and has only spread out of that region in recent times, then it could still be showing signs of continued outward spread out of Asia. To examine this possibility we surveyed for its presence in *A. cerana* and *A. mellifera* in the Solomon Islands, where both bees are not indigenous, but where the situation lends itself to an examination of a possible recent invasion of *A. mellifera* by *N. ceranae* carried by *A. cerana* that originated from Asia. The Solomon Islands is a nation of more than 900 islands located to the east of Papua New Guinea. Since 2000, some of these islands have become colonized by a Java form of *A. cerana* that has become an invasive pest in the region following its initial introduction into neighboring New Guinea from Java, Indonesia by humans (Anderson, 1994; Annand, 2008). Some of the islands have also been inhabited by *A. mellifera*, artificially introduced by humans from New Zealand and Australia more than 30 years ago (Reid and Van Eaton, 1993), at a time when *N. ceranae* was not present in *A. mellifera* in either country, as determined by rDNA gene sequence studies (Gatehouse et al., 1999; Malone et al., 1994; Rice, 1999, 2001). Hence, it was possible to survey for *N. ceranae* on separate islands where *A. mellifera* and *A. cerana* occurred alone or sympatric. To confirm previous reports that *N. ceranae* is possibly widespread in Asia (Fries et al., 1996; Chaimanee et al., 2010) we surveyed for its presence in *A. cerana* populations throughout the Philippines, Indonesia, Sri Lanka, India, Thailand, Vietnam, South Korea, China and Papua New Guinea, placing emphasis on Indonesia, as it had not been previously surveyed for the parasite and it was also the origin of the Asian bees now found in the Solomon Islands. We also surveyed for *N. ceranae* in *A. koschevnikovi* from Borneo, in *A. nigrocincta* from Sulawesi and in *A. florea* from Vietnam and Thailand.

## 2. Materials and methods

### 2.1. Evaluating possible recent spread of N. ceranae out of Asia

To evaluate a possible recent cross infection of *N. ceranae* from *A. cerana* to *A. mellifera*, a total of 25 *A. mellifera* and 11 *A. cerana* samples were collected from seven separate islands of the Solomon Islands archipelago. Each sample consisted of more than 30 adult bees. On each island sampled, except on Savo, Guadalcanal and Malaita Island, the resident *A. mellifera* were sympatric with the recently invaded *A. cerana*. On Savo and Guadalcanal Islands, *A. cerana* had previously been sympatric with *A. mellifera*, but at the time of sampling, those islands were totally free of *A. mellifera*. The *A. mellifera* on Malaita Island had never been in contact with *A. cerana* or any other species of *Apis*. Collected samples were stored at −18 °C in ethanol until the bee species were unequivocally identified along with the type of *Nosema* species they were infected with.

Bee species were determined using taxonomic keys, morphological characteristics and from DNA sequence obtained from a region of the ribosomal CO-II gene region. For this, DNA was first extracted from individual bees using JETQUICK Tissue DVA Spin Kits®, as instructed by the manufacturer. A region of the CO-II gene was amplified using an Eppendorf Mastercycler® a forward primer (5′-AATTGATGAAATTGTAAATC-3′) and reverse primer (5′-GGCCGTCTGACAAACTAATGTTAT-3′) as described by Anderson and Morgan (2007). Amplified DNA was sequenced using a CEQTM 8000 Genetic Analysis System (Beckman Coulter) in combination with the specific PCR primers and CEQTM Dye Terminator Cycle Sequencing with Quick Start Kits (Beckman Coulter), as per the manufacturer's instructions. DNA sequences were then compared with those in Genbank as well as with other similar sequences obtained from Asian honeybees across the Asian region (Anderson, unpublished data).

The presence and identity of *Nosema* spp., in the various *A. cerana* and *A. mellifera* samples were determined by DNA analysis. First, the abdomens were removed from each of 30 to 50 adult worker bees in each sample and macerated in 10 ml. of distilled water with a mortar and pestle. The resulting homogenates were then purified by centrifugation for 6 min at 800*g*. (OIE, 2008) and the pellets re-suspended in 500 μl of distilled water in a 1.5 ml Eppendorf tube. Subsequently, 0.1 g. of glass beads (1.0 mm diameter) were added to each tube and vortexed at 3000 rpm for 1 min. DNA extraction was done as described previously (Higes et al., 2008a). The presence and absence of *N. apis* and *N. ceranae* DNA in each DNA preparation was determined by PCR using the 321APIS and 218MITOC primers (Martín-Hernández et al., 2007), performed by a Mastercycler© gradient (Eppendorf) in a reaction volume of 50 μl containing 5 μl of 10× CoralLoad PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.4 μM of each primer, 0,1% Triton X-100, 0,2 mg/ml bovine serum albumin, 0.25 μl of Taq DNA Polymerase (Taq PCR Core Kit, catalog No. 201223; QIAGEN) and 5 μl of DNA template. Cycling conditions were as follows: Initial denaturation step of 3 min. at 94 °C, followed by 30 cycles of 45 s. at 94 °C, 45 s. at 61.8 °C, and 1 min. at 72 °C and a final extension step at 72 °C for 10 min. Negative and positive controls were included in the DNA extraction process and PCR experiments to detect possible contaminations and check the reliability of the processing of the samples. PCR products were analyzed on a 1.5% agarose gel with ethidium bromide to visualize DNA bands.

### 2.2. Verifying the extent of spread of N. ceranae in Asia

To verify the extent of spread of *N. ceranae* in Asia, a total of 45 samples of adult bees of either *Apis cerana* ($N = 38$), *Apis florea* ($N = 3$), *Apis nigrocincta* ($N = 2$) and *Apis koschevnikovi* ($N = 2$) were collected into 70% (v/v) ethanol from 1991 to 2004 from locations shown in Fig. 1. Each sample consisted of more than 10 adult bees and was stored at −18 °C in ethanol until the identify of the bees and the type of *Nosema* they were infected with were determined, by methods described above.

*N. ceranae* positive PCR products detected in samples of *A. cerana* collected from Vietnam and South Korea, and from *A. koschevnikovi* from Borneo were further purified and sequenced in both directions as described by Martín-Hernández et al. (2007) in order to confirm that the PCR products corresponded to *N. ceranae* and to study and compare sequences.

## 3. Results

### 3.1. Bee samples from the Solomon Islands

*N. ceranae* was the only microsporidium detected in honeybees in the Solomon Islands. However, it was only present in bee samples collected from Islands that had been invaded by *A. cerana*. On those islands it was present in both *A. mellifera* and *A. cerana* (Table 1; Fig 1). It was not detected in 12 *A. mellifera* samples

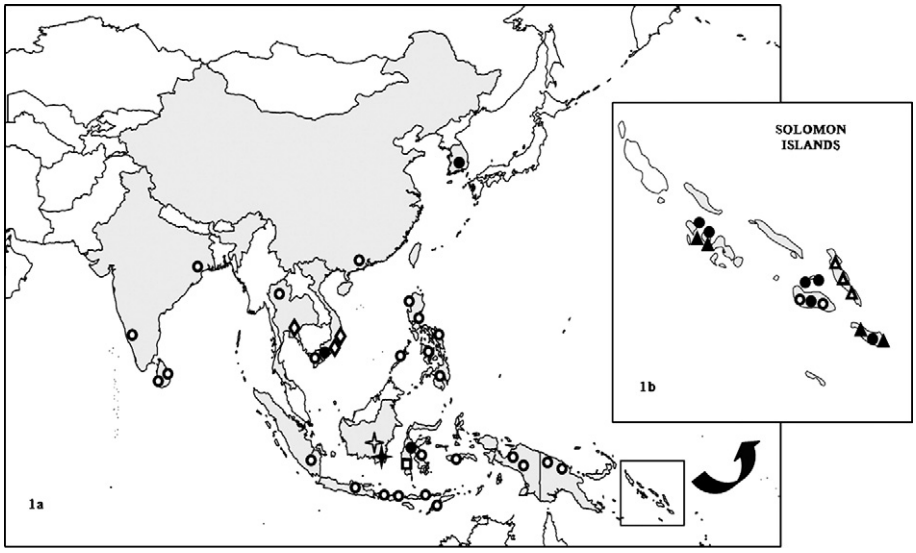


**Fig. 1.** Distribution of *Apis* spp., examined for *N. ceranae* in this study: *A. mellifera* (triangle), *A. cerana* (circle), *A. koschevnikovi* (star), *A. nigrocincta* (square), *A. florea* (rhombus). Filled and empty symbols represent those bee samples that contained or were free of *N. ceranae* respectively. Inset shows the presence and absence of *N. ceranae* in samples of *A. mellifera* and *A. cerana* collected from the Solomon Islands.

**Table 1**
The location, date of sampling and host species of the *Nosema* positive samples based on PCR analysis.

| Country | Sampling date | Host species | Region/Prov./Island | *Nosema* species detected by PCR |
|---|---|---|---|---|
| Indonesia | 24/08/2004 | *A. cerana* | SULAWESI | *N. ceranae* |
| | 26/08/2004 | *A. koschevnikovi* | SOUTH KALIMATAN | *N. ceranae* |
| Vietnam | 07/08/2004 | *A. cerana* | TIEN GIANG | *N. ceranae* |
| South Korea | 19/10/1996 | *A. cerana* | | *N. ceranae* |
| Solomon Islands | 11/11/2008 | *A. cerana* | KOLOMBANGARA | *N. ceranae* |
| | 11/11/2008 | *A. mellifera* | KOLOMBANGARA | *N. ceranae* |
| | 11/11/2008 | *A. mellifera* | KOLOMBANGARA | *N. ceranae* |
| | 11/11/2008 | *A. mellifera* | KOLOMBANGARA | *N. ceranae* |
| | 11/11/2008 | *A. mellifera* | KOLOMBANGARA | *N. ceranae* |
| | 11/11/2008 | *A. mellifera* | KOLOMBANGARA | *N. ceranae* |
| | 2009 | *A. mellifera* | KOLOMBANGARA | *N. ceranae* |
| | 20/11/2008 | *A. cerana* | GUADALCANAL | *N. ceranae* |
| | 19/11/2008 | *A. cerana* | SAVO | *N. ceranae* |
| | 20/05/2009 | *A. cerana* | SAVO | *N. ceranae* |
| | 20/05/2009 | *A. cerana* | SAVO | *N. ceranae* |
| | 18/05/2009 | *A. cerana* | MAKIRA | *N. ceranae* |
| | 19/05/2009 | *A. mellifera* | MAKIRA | *N. ceranae* |
| | 18/05/2009 | *A. mellifera* | MAKIRA | *N. ceranae* |
| | 2009 | *A. cerana* | GIZO | *N. ceranae* |
| | 2009 | *A. mellifera* | GIZO | *N. ceranae* |
| | 2009 | *A. mellifera* | GIZO | *N. ceranae* |
| | 2009 | *A. mellifera* | GIZO | *N. ceranae* |
| | 2009 | *A. mellifera* | GIZO | *N. ceranae* |
| | 12/05/2009 | *A. cerana* | TULAGI | *N. ceranae* |
| | 12/05/2009 | *A. cerana* | TULAGI | *N. ceranae* |

collected from Malaita Island which has never been invaded by *A. cerana* or any other species of *Apis* (Fig 1). The percentages of *N. ceranae* in *A. cerana* and *A. mellifera* samples collected throughout the Solomon Islands is summarized in Table 2.

### 3.2. Bee samples from south-east Asia

*N. ceranae* was the only microsporidium detected in the honeybees collected from south-east Asia. It was detected in one of 14 samples of *A. cerana* collected from Indonesia (Sulawesi Island), one of 4 from Vietnam and in a single sample collected from South Korea (Table 1). It was also detected in one of two samples of *A. koschevnikovi* collected from Borneo. It was not detected in any samples of *A. florea* ($N = 3$) or *A. nigrocincta* ($N = 2$).

The consensus sequences for the 16S ribosomal RNA gene of *N. ceranae* found in *A. cerana* collected from South Korea and Vietnam and in *A. koschevnikovi* collected from Borneo have been deposited in the GenBank database (accession numbers FJ78980, FJ789800 and FJ789802 respectively).

## 4. Discussion

*N. ceranae* was first confirmed as a parasite of honeybees (*Apis* spp.) during the mid 1990s, but only following the development of rDNA gene marker technology for identifying microsporidia of honeybees (Fries et al., 1996). Initially, it was detected in the Asian honeybee (*A. cerana*) in Asia and then subsequently in Western

**Table 2**
Number of samples of each bee species tested (*N*) from the total known number of hived colonies present [shown in square brackets], *N. ceranae* positive samples (*n*) and percentage of positive samples (%) in each territory analyzed from the Solomon Islands.

| Island | Bee species (Host) | | | | | |
|---|---|---|---|---|---|---|
| | *Apis cerana* | | | *Apis mellifera* | | |
| | *N* | *n* | % | *N* | *n* | % |
| Malaita | – | – | – | 12[800] | 0 | 0 |
| Guadalcanal | 3 | 1 | 33.3 | – | – | – |
| Makira | 1 | 1 | 100 | 2[12] | 2 | 100 |
| Savo | 2 | 2 | 100 | – | – | – |
| Tulagi | 2 | 2 | 100 | – | – | – |
| Gizo | 1 | 1 | 100 | 4[24] | 4 | 100 |
| Kolombangara | 2 | 1 | 50 | 7[38] | 6 | 85.7 |
| Total | 11 | 8 | 72.7 | 25[874] | 12 | 48 |

honeybees (*A. mellifera*) almost globally. Since its discovery a diverse body of evidence has built up that suggests that the parasite may have originally parasitized Asian honeybees and that it has only recently become a parasite of *A. mellifera* (Cornman et al., 2009; Fries et al., 2006; Giersch et al., 2009; Higes et al., 2006; Klee et al., 2007; Martín-Hernández et al., 2007).

Even though *N. ceranae* was first isolated from *A. cerana* in Asia, the task of determining its primary host was made difficult when it was subsequently found to be widespread in *A. mellifera* (Invernizzi et al., 2009; Klee et al., 2007; MAF New Zealand, 2010; Soroker et al., 2011; Yoshiyama and Kimura, 2011) and also present in several different bee species (Chaimanee et al., 2010; Plischuk et al., 2009; Suwannapong et al., 2010a). If *N. ceranae* originated in Asia and has only recently spread out of Asia, then it could still be showing signs of its ongoing spread out of Asia and it should be fairly widespread in Asia, as suggested by previous studies. Our studies in the Solomon Islands found compelling evidence that *N. ceranae* is still showing signs of spreading out of Asia and we also verified the findings of other authors that the parasite is widespread in Asian honeybees.

The situation in the Solomon Islands lent itself to a study of the continual on-going spread of *N. ceranae* out of Asia. That island country is not home to any native *Apis* species. However, humans have introduced *A. mellifera* to many islands from New Zealand and Australia, with the last imports being more than 30 years ago (Reid and Van Eaton, 1993; S. Ete Solomon Islands Department of Agriculture and Livestock, personal communication). This was at a time when *N. ceranae* was not present in *A. mellifera* in New Zealand or Australia, as confirmed using rDNA gene markers that were capable of detecting *N. ceranae* (Gatehouse et al., 1999; Malone et al., 1994; Rice, 1999, 2001). Hence, the *A. mellifera* colonies in the Solomon Islands should also be totally free of *N. ceranae*. Since the early 2000s some islands of the Solomon Islands have also become colonized with *A. cerana*, which spread into the region from New Guinea where, in turn, the bee had become invasive following its initial introduction from Java Indonesia by humans during the 1970s (Anderson, 1994). Not all islands in the Solomon Islands are yet colonized by *A. cerana* and therefore it was possible for us to survey for *N. ceranae* on islands where *A. mellifera* and *A. cerana* occurred alone or sympatric. On islands where the bees were sympatric, *N. ceranae* was infecting both species. On islands only inhabited with *A. cerana* (Savo and Guadalcanal Islands, Table 1), *N. ceranae* was found infecting those bees. On Malaita Island, which had not yet been invaded by *A. cerana* (Annand, 2008; Anderson, unpublished data), *N. ceranae* was not detected in the resident *A. mellifera*. Therefore, the absence of *N. ceranae* infection in the *A. mellifera* samples from Malaita Island and its presence in *A. mellifera* from other Islands where this bee species is sympatric with *A. cerana* gives consistent evidence of the recent introduction of *N. ceranae* into the Solomon Islands by *A. cerana*.

If *A. cerana* introduced *N. ceranae* into the Solomon Islands, as our results indicate, then the question remains as to whether the *A. cerana* that recently entered the Solomon Islands had contact with *A. mellifera* infected with *N. ceranae* before they entered the Solomon Islands. DNA evidence indicates that *A. cerana* in the Solomon Islands originated from neighboring New Guinea where, in turn, it had been imported by humans from Java during the 1970s (Anderson, 1994). That importation occurred at about the same time (1975) that beekeeping with *A. mellifera* was initiated in Java with stock imported from Australia (Moncur et al., 1995), and hence those *A. mellifera* would have been free of *N. ceranae*. Following the introduction of *A. cerana* into the western half of New Guinea (Irian Jaya) from Java, it became invasive and spread through the entire island. As it spread it came into contact with *A. mellifera* in both Irian Jaya and Papua New Guinea. However, the *A. mellifera* in those regions were descendent from stock introduced from Australia and New Zealand before the early 1970s (Clinch, 1979) and therefore would have been totally free of *N. ceranae*. Hence, there is no evidence that the *A. cerana* we tested in the Solomon Islands had prior contact with *A. mellifera* infected with *N. ceranae* and therefore almost certainly introduced *N. ceranae* to *A. mellifera* in the Solomon Islands.

We found *N. ceranae* to be widespread in Asian honeybees. Not only did we find it in *A. cerana* from north, central and south Asia (Korea, Vietnam and Indonesia respectively), but we also report it for the first time from *A. koschevnikovi* from Borneo. In our studies, no *Nosema* species were detected in samples of *A. florea* from Vietnam ($N = 3$) and *Apis nigrocincta* from Indonesia ($N = 2$). Nevertheless, *N. ceranae* has been previously reported from *A. florea* in Thailand (Suwannapong et al., 2010a) and thus, given our limited sample size of *A. nigrocincta*, we cannot exclude the possibility of that bee being a potential host for *N. ceranae*. Indeed, this is very likely, given that *A. nigrocincta* is sympatric with *A. cerana* in Sulawesi (which we showed carries *N. ceranae*) and *N. ceranae* has been shown to infect a diverse group of *Apis* and non-*Apis* pollinators (Smith et al., 2000; Plischuk et al., 2009; Chaimanee et al., 2010).

The complete absence of *N. apis* in bee samples we analyzed from Asia and the Solomon Islands warrants further discussion. Possibly the general low prevalence of *N. apis* in Asia (Chaimanee et al., 2010; Chen et al., 2009; Suwannapong et al., 2010a; Yoshiyama and Kimura, 2011) explains why we failed to detect it in the limited number of samples we examined from that region. However, its absence in samples we examined from the Solomon Islands, particularly in the *A. mellifera* samples, is not so clear. Perhaps *N. ceranae* may have displaced *N. apis* when islands became colonized by *A. cerana* carrying *N. ceranae*. Nevertheless *N. apis* was also absent in the reasonably large number of *A. mellifera* samples we examined from Malaita Island, which is free of *A. cerana*. Perhaps the *A. mellifera* that were originally imported to that island were totally free of *Nosema*. This possibility warrants further investigation.

The mechanism by which *N. ceranae* broadened its host range from an Asian bee species to the Western honey bee in different parts of the world is unknown, but there has now been contact between Asian honeybees and *A. mellifera* for possibly more than a century, mediated by humans, before which there was complete separation (Oldroyd and Nanork, 2009; Ruttner and Maul, 1983; Sakai and Okada, 1973). Moreover, there are reports of *A. cerana indica* being imported into Europe from Pakistan in 1968 for scientific research purposes (Ruttner and Kaissling, 1968 (German) revised by Koeniger and Koeniger (2000). Otherwise, the importation of pollen of Asian origin to the Western world in order to fulfill the increasing demand of this source to rear commercial pollina-

tors may have played a role in the spread of this microsporidium (H.H.W. Velthuis, personal communication), as pollen has been shown to hold infective *N. ceranae* spores (Higes et al., 2008b). Nevertheless, this hypothesis should be further investigated. Our results shed some light on the invasion process and suggest that *N. ceranae* may have originated in Asia to become a highly prevalent pathogen of *A. mellifera* as also suggested previously by other authors (Higes et al., 2006; Klee et al., 2007; Cornman et al., 2009). Perhaps future phylogenetic analysis based on comparable gene sequence from several *N. ceranae* strains from different locations and hosts may provide further insight as to the origin and dispersal routes of *N. ceranae* and help clarify how long it has been present in Western countries.

Finally, honey bees are major pollinators of plants and crops in terrestrial ecosystems, so their possible population decline from the effects of microbial pathogens and other agents is of importance for humans and biodiversity (Brown and Paxton, 2009). Our results call for efforts to better understand the nature of host–parasite interactions and the mechanisms that enable or confine successful parasitism. A knowledge of the original host of *N. ceranae* and the interactions between the two may help identify possible interspecific co-adaptations that may have been acquired by decades of parasitism and that may open new approaches for the control of nosemosis type C in *A. mellifera*, caused by *N. ceranae*.

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# Artículo 4

## Abejorros (Hymenoptera: Apidae) autóctonos sudamericanos infectados por *Nosema ceranae* (Microsporidia), agente etiológico de una enfermedad emergente en las abejas melíferas (*Apis mellifera*)

Cristina Botías, Raquel Martín-Hernández, Encarna Garrido-Bailón, Amelia González-Porto, Amparo Martínez-Salvador, Pilar De la Rúa, Aránzazu Meana y Mariano Higes.

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*Debido a que la polinización es un proceso crucial tanto para los ecosistemas terrestres humanizados como para los naturales, los polinizadores proporcionan un servicio esencial tanto para la naturaleza como para los seres humanos. La polinización se debe principalmente a la acción de diferentes insectos, tales como los abejorros y las abejas. El papel clave que desarrollan estos organismos ha llevado a una preocupación muy extendida acerca de la reciente pérdida de poblaciones de polinizadores, la cual se ha detectado en diversas regiones del mundo. Mientras que este mencionado descenso se ha atribuido en algunos casos a los cambios sufridos en los usos de los terrenos agrícolas, también los efectos provocados por los parásitos podrían jugar un papel importante en la reducción de estas poblaciones.*

*En este estudio se describe por primera vez la presencia de* Nosema ceranae*, agente etiológico de una enfermedad emergente de las abejas melíferas, en tres especies autóctonas de abejorros argentinos. Para ello se analizaron un total de 455 abejorros procedentes de seis especies del género* Bombus*. Los resultados hallados tras realizar el análisis por PCR mostraron resultados positivos a la presencia de N. ceranae en tres de las seis especies examinadas (*Bombus atratus*,* Bombus morio *y* Bombus bellicosus*).*

*En este estudio se discute la aparición de este patógeno en el contexto de la disminución en las poblaciones de estos polinizadores.*

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# South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (*Microsporidia*), an emerging pathogen of honeybees (*Apis mellifera*)

**Santiago Plischuk,[1] Raquel Martín-Hernández,[2] Lourdes Prieto,[3] Mariano Lucía,[4] Cristina Botías,[2] Aránzazu Meana,[5] Alberto H. Abrahamovich,[4] Carlos Lange[1,6] and Mariano Higes[2]***

[1]*Centro de Estudios Parasitológicos y de Vectores (CEPAVE), CIC – CCT La Plata – CONICET – UNLP, calle 2 # 584, (1900), La Plata, Argentina.*

[2]*Bee Pathology Laboratory, Centro Apícola Regional, JCCM, 19180 Marchamalo, Spain.*

[3]*Instituto Universitario de Investigación en Ciencias Policiales (IUICP). Comisaría General de Policía Científica (Forensic Police). DNA Laboratory, Madrid, Spain.*

[4]*CONICET-UNLP. Laboratorio de Apidología, División Entomología, Museo de La Plata, Paseo del Bosque s/n, 1900, La Plata, Argentina.*

[5]*Animal Health Department, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.*

[6]*Comisión de Investigaciones Científicas (CIC), Buenos Aires province, Argentina.*

## Summary

**As pollination is a critical process in both human-managed and natural terrestrial ecosystems, pollinators provide essential services to both nature and humans. Pollination is mainly due to the action of different insects, such as the bumblebee and the honeybee. These important ecological and economic roles have led to widespread concern over the recent decline in pollinator populations that has been detected in many regions of the world. While this decline has been attributed in some cases to changes in the use of agricultural land, the effects of parasites could play a significant role in the reduction of these populations. For the first time, we describe here the presence of *Nosema ceranae*, an emerging honeybee pathogen, in three species of Argentine native bumblebees. A total of 455 bumblebees belonging to six species of genus *Bombus* were examined. PCR results showed that three of the species are positive to *N. ceranae* (*Bombus atratus*, *Bombus morio* and *Bombus bellicosus*). We discuss the appearance of this pathogen in the context of the population decline of this pollinators.**

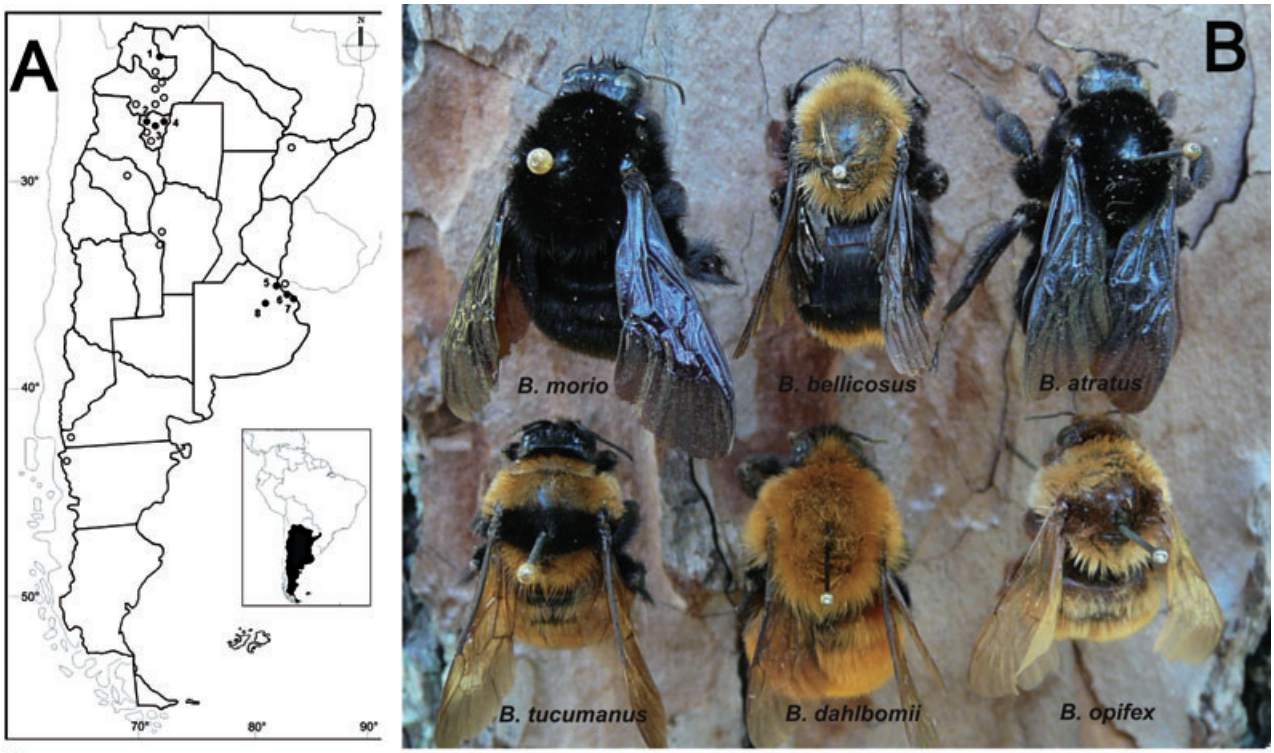
Received 23 November, 2008; accepted 16 January, 2009. *For correspondence. E-mail mhiges@jccm.es; Tel. (+34) 949 250 026; Fax (+34) 949 250 176.



## Introduction

Bumblebees and honeybees are pollinators that fulfil an essential duty into nature (Goulson, 2003; Klein *et al*., 2007) since pollination is critical for food production and by continuation for human livelihood. Indeed it is a process that directly links natural ecosystems with agricultural production systems (Klein *et al*., 2007; FAO, 2008). Bumblebees are, in general, annual social insects. There are some 240 species of genus *Bombus* within the family Apidae, the majority of which are polilectic, visiting a wide diversity of plants (Moure and Sakagami, 1962; Prouveau, 1984; Abrahamovich and Díaz, 2001). Most of them are distributed in the temperate areas of North America and Eurasia, in part because they are able to forage under cool and rainy conditions. In the neotropical and Andean regions, only 42 species have been recorded in a wide variety of habitats, from sea level up to about 4400 m in the Andes. In Argentina, only eight neotropical species have been described and *Bombus atratus*, *Bombus morio* and *Bombus bellicosus* (Fig. 1B) display the most widespread distribution. The first species is the most abundant in Argentina, probably due to its tolerance of different climates and altitudes.

Commercial rearing of bumblebee colonies for pollination commenced in the 1980s in Europe (Banda and Paxton, 1991; Van den Eijnde *et al*., 1991) which led to the spreading of these famed insects worldwide. This spread was associated with the inherent risk of exporting and introducing different diseases into new regions where adequate sanitary control at the frontiers is not maintained. Given the ecological and economic importance of these insects there is now widespread concern over the recent decline in pollinator populations that has been detected in many regions of the world, both in *Apis mellifera* and in bumblebees (Williams, 2005; FAO, 2008).




C

| Bumblebee species | N | Nosema-like posiitve (n = 53) PCM | % | *Nosema ceranae* (n = 53) PCR | % | *Nosema apis* (n=53) PCR | *Nosema bombi* (n = 53) PCR |
|---|---|---|---|---|---|---|---|
| *B. atratus* | 368* (239W, 96Q, 4M, 29I) | 50 (45W, 4Q, 1M) | 13.6 | 46 (41W, 4Q, 1M) | 12.5 | 0 | 0 |
| *B bellicosus* | 16 (10W, 6Q) | 1 (1W) | 6.25 | 1 (1W) | 6.25 | 0 | 0 |
| *B. dahlbomii* | 15 (3W, 2Q, 3M, 7I) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. morio* | 42 (16W, 25Q, 1M) | 2 (2Q) | 4.76 | 2 (2Q) | 4.76 | 0 | 0 |
| *B. opifex* | 8 (6W, 2Q) | 0 | 0% | 0 | 0 | 0 | 0 |
| *B. tucumanus* | 6 (4W, 2Q) | 0 | 0% | 0 | 0 | 0 | 0 |

**Fig. 1.** A total of 455 bumblebees were studied (21 of them sampled in 1987). Bumblebees were sampled by collecting individuals in entomological nets between March 2005 and May 2008 in different regions of Argentina. Each one was identified and classified with taxonomic keys described by Abrahamovich and colleagues (2005). The bumblebees collected were conserved in 70% ethanol or frozen until they were analysed. The samples collected in 1987 were kept in absolute ethanol. The bumblebees were dissected and the microsporidia-like spores they contained were counted by phases-contrast microscopy (PCM) and the specie determined by PCR (described below). To detect microsporidia-like spores, whole bumblebees were generally homogenized individually in 2 ml of distilled water, and the fresh preparations from the resulting homogenates were observed by PCM (X400, X1000). Purified spore suspensions were prepared from macerates that were identified as positive for microsporidia-like spores by PCM, and the suspensions were filtered (55 μm mesh) and centrifuged. Dissection of bumblebee was made as described (Larsson, 2007). W, worker bumblebee; Q, queen bumblebee; M, male bumblebee; I, immature bumblebee. Percentages are calculated in the basis of the number of each bumblebee species analysed.
A. Collection sites in Argentina where bumblebees were collected. Solid circles: *N. ceranae* positive. Empty circles: *N. ceranae* negative. 1, Calilegua (Jujuy province); 2, El Manantial; 3, Tafí viejo; 4, La Reducción (2, 3 and 4 from Tucumán province); 5, San Miguel; 6, Punta Lara; 7, La Plata; 8, Lobos (5, 6, 7 and 8 from Buenos Aires province).
B. Native bumblebee species from Argentina included in this work.
C. Table summary of the analysis results of PCM and PCR in bumblebees. The asterisk (*) indicates the 21 bumblebees sampled in 1987.

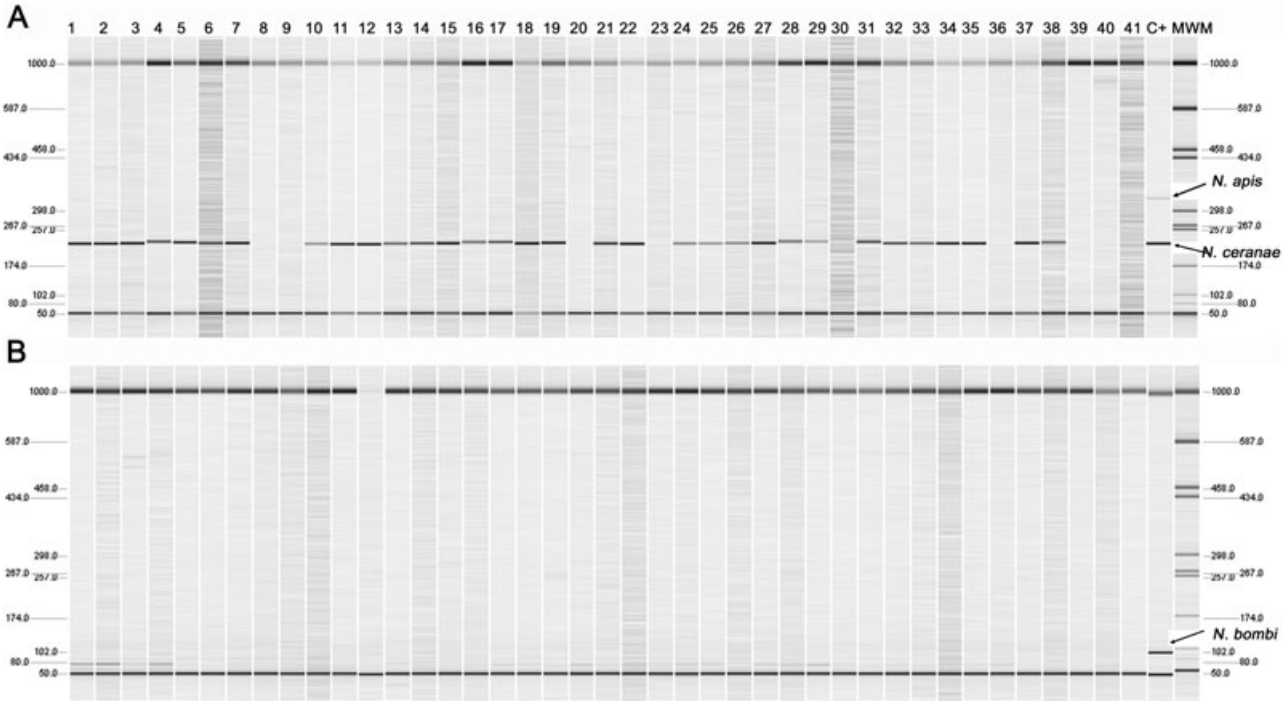



**Fig. 2.** *Nosema apis* or *N. ceranae* species were determined as previously described (Martín-Hernández *et al*., 2007; Higes *et al*., 2008), using the 321APIS and 218MITOC primers. *Nosema bombi* determination was made using BOMBICAR primers, designed in this work. The rRNA locus was selected to search *N. bombi* published sequences in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and 62 accession numbers (see Appendix S1 in *Supporting information*) were selected, excluding those sequences that contained a high number of ambiguities (Ns). Sequences were aligned by using CLUSTALW (http://www.ebi.ac.uk/clustalw/) in order to identify individual polymorphic nucleotide positions and to avoid them as primer-binding zones. A consensus sequence (with variable sites, naming them by using IUB code) was obtained for *N. bombi*. Specific primers for *N. bombi* were visually selected taking into account that the primer sequences were specific to this specie. The selected primers were: BOMBICAR-F 5′-**GG**CCCATGCATGTTTTTGAAGATTATTAT-3′ (5′-GG tail added to the primer is underlined and in bold) and BOMBICAR-R 5′-CTACACTTTAACGTAGTTATCTGCGG-3′, expected to produce an amplicon of 101 bp. Species specificity was performed doing a nearly exact match searching with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Once the specific annealing of *N. bombi* primers was verified, the optimal annealing temperature was set at 58.1°C. All the PCRs reactions were carried out in a Mastercycler® ep gradient S (Eppendorf) in a volume of 50 μl using the High Fidelity PCR Master (No. 12140314001 Roche Diagnostic), 0.4 μM of each primer, 0.2 mg $ml^{-1}$ BSA, 0.1% Triton X-100 and 5 μl of DNA template. Each PCR was analysed in QIAxcel System (Qiagen), using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002). Negative controls were analysed in parallel to detect possible contaminations. Sequencing of PCR products from one *N. bombi* and three *N. ceranae* positive samples was performed in order to ensure that products with the correct sequence and size were generated. The figure shows the same 41 samples analysed for *N. apis*/*N. ceranae* (A) and for *N. bombi* (B). As positive controls (C+), DNA template either from *N. bombi* (supplied by Dr. Paxton) or from an *Apis mellifera* sample positive to *N. apis* and *N. ceranae* were used. MWM, molecular weight marker. Lines at 50 and 1000 bp correspond with the alignment marker (Qiagen).

The causes of this decline are thought to be related to changes in the use of agricultural land (Williams, 2005) but also, to the effects of parasites (Otti and Schmid-Hempel, 2007). Indeed, devastating losses have been observed in honeybee colonies (*A. mellifera*) over recent years, the other major pollinator (Stokstad, 2007; Molga, 2008) and recent reports of global pollinator decline justify the search for activities that help to conserve them (FAO, 2008).

Although the cause of honeybee losses is not clear, pathogens seem to play a central role in this phenomenon (Martín-Hernández *et al*., 2007; Blanchard *et al*., 2008; Higes *et al*., 2008; Van Ooij, 2008). Bumblebees host a highly pathogenic species of microsporidia, *Nosema bombi*, described by Fantham and Porter (1914), currently the only microsporidia known to infect bumblebees (Tay *et al*., 2005). This pathogen reproduces primarily in the Malpighian tubules (Fries *et al*., 2001) and it extends secondarily into the midgut, fat body and other organs (Larsson, 2007).

In this work, the first identification of *Nosema ceranae* in bumblebees is reported.

## Results and discussion

Here we describe the first identification of *N. ceranae* in bumblebee, until now considered a parasite of *A. mellifera* and *Apis cerana* (Fries *et al*., 1996; Higes *et al*., 2006; Huang *et al*., 2007) that has been associated with bee death following experimental or natural infection (Higes *et al*., 2007; 2008; Paxton *et al*., 2007).

*Microsporidia*-like spores were observed in 53 samples [phases-contrast microscopy (PCM) analysis]

and *N. ceranae* was PCR confirmed in 49 out of these 53 samples (Fig. 1C). The consensus sequence obtained form *N. ceranae* was submitted to GenBank (Accession No. FJ227957). *Nosema* spp. were not detected by PCM and PCR methods in any of the samples from the collection that dated from 1987. Likewise *N. bombi* and *N. apis* were not detected in any sample (Figs 1C and 2).

Spores of *N. ceranae* were only detected in the ventriculus that had been dissected out from the bumblebees and spores from this microsporidium were not detected in Malpighian tubules or fat bodies when analysed separately by PCM. The ventriculus parasitized by *N. ceranae* developed similar macroscopical lesions to those described in infected bees (Fries *et al*., 1996; Higes *et al*., 2007; 2008). Moreover, mature spores of *N. ceranae* were observed in high quantities in all castes of the *Bombus* species (Fig. 1C).

From *B. atratus* mature spores of *N. ceranae* were detected in samples from workers ($8.05 \times 10^6 \pm 1.44 \times 10^6$; spores per bumblebee, mean $\pm$ SE; $n = 45$), a male sample ($3.15 \times 10^5$; $n = 1$) and queens ($5.76 \times 10^6 \pm 1.38 \times 10^6$; $n = 4$). In contrast, only one *B. atratus* worker was positive ($1.14 \times 10^7$; $n = 1$), as were only two *B. morio* queens ($1.66 \times 10^8$ and $1.1 \times 10^8$, $n = 2$). When considered in conjunction these molecular and quantitative data and the lesions macroscopically detected in ventriculus suggest that *N. ceranae* can parasitize individuals of all bumblebee castes in this country. Experimental infections and subsequent pathological studies would clear the real pathogenicity of this microsporidium to bumblebees and their consequences.

*Nosema ceranae* has been detected in two quite distant regions implying a wide dispersion of this pathogen. There are lot of honeybee colonies in both regions and in the southernmost (Pampas region), and this microsporidium has recently been identified in colonies suffering significant colony loss (Plischuk *et al*., 2008). Indeed, bumblebees might have acquired this infection through their close contact with these colonies, or vice versa, and the role of other pollinators in such infections cannot be undermined.

The findings described here are somewhat worrying due first to the wide geographic spread of *Bombus* which enhances their chances of encountering parasites and, therefore, of being colonized. Second, the farming and commerce of species without the use of adequate diagnostics tools to ensure the absence of this pathogen may lead to its widespread dissemination of parasites. And third, if the pathogenicity of *N. ceranae* in bumblebees is later confirmed by experimental studies, the consequences on survival of these pollinator population should be evaluated in order to get quick and reliable control measures to guarantee their conservation.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** A new PCR from *Nosema bombi*.

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# Artículo 5

## Presencia de *Nosema ceranae* (Microsporidia) en abejas norteafricanas (*Apis mellifera intermissa*)

Mariano Higes, Raquel Martín-Hernández, Encarna Garrido-Bailón, Cristina Botías y Aránzazu Meana

*Journal of Apicultural Research and Bee World (2009) 48(3): 217-219*

*Hasta el momento, únicamente dos especies de microsporidios se han descritos como patógenos de* Apis mellifera: Nosema apis *y* Nosema ceranae*. Mientras que la infección por Nosema apis ha sido ampliamente descrita y ha sido detectada en poblaciones de abejas de todos aquellos lugares en los que se practica la apicultura con Apis mellifera, las infecciones provocadas por* N. ceranae *en la abeja melífera no se han conocido hasta hace unos años. Este microsporidio se considera en la actualidad como un patógeno de consecuencias graves para* A. mellifera*, habiéndose asociado a un síndrome con un despoblamiento gradual de colonias. Hasta el momento este patógeno se ha encontrado en poblaciones de* A. mellifera *de cuatro continentes: Asia, Europa, Norteamérica y Sudamérica, pero no se han encontrado evidencias sobre su presencia en los países de África.*

*En este estudio se analizaron un total de 24 muestras de abejas obreras, las cuales procedían de 12 colonias seleccionadas al azar (2 muestras recogidas de cada colonia). Estas colmenas se encontraban situadas dentro de en un colmenar próximo a la localidad de Sidi bel Abbes (Argelia), en el cual se estaban experimentando grandes pérdidas de colmenas, con una inexplicable desaparición de abejas pecoreadoras en las mismas.*

*Los únicos agentes patógenos detectados en las muestras analizadas fueron los microsporidios del género Nosema. En concreto,* N. ceranae *fue detectada en 8 de las 24 muestras recogidas (6 muestras positivas de tres colonias colapsadas y dos procedentes de una colonia aparentemente sana). Por su parte,* N. apis *fue únicamente encontrada en una muestra de abejas procedente de una colonia desprovista de signos clínicos de la enfermedad.*

*Esta es la primera vez que* N. ceranae *se detecta en colonias de* Apis mellifera intermissa *con signos de despoblamiento en el continente africano, lo cual aporta pruebas sobre la amplia distribución de este patógeno y sobre el incremento incipiente en su prevalencia en las colonias de abejas melíferas a nivel mundial.*

***PRESENTACIÓN EN CONGRESOS:***

*Higes M., Martín-Hernández R., Garrido-Bailón E., Botías C., Meana A. The presence of* Nosema ceranae *(Microsporidia) in North African honey bees (*Apis mellifera intermissa*). 41º Congreso APIMONDIA, 15-20 de Septiembre 2009, Montpellier (Francia).*

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NOTES AND COMMENTS

# The presence of *Nosema ceranae* (Microsporidia) in North African honey bees (*Apis mellifera intermissa*)

**Mariano Higes*[1], Raquel Martín-Hernández[1], Encarna Garrido-Bailón[1], Cristina Botías[1] and Aránzazu Meana[2]**

[1]Centro Apícola (CAR), Bee Pathology Laboratory, Junta Comunidades Castilla-La Mancha, 19180 Marchamalo, Spain.
[2]Facultad de Veterinaria, Universidad Complutense. 28040 Madrid, Spain.

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*Corresponding author: Email: mhiges@jccm.es

**Key words:** *Apis mellifera intermissa*, *Nosema ceranae*, epidemiology, colony depopulation, African countries

Until now, only two species of microsporidia have been described to infect *Apis mellifera*: *Nosema apis* and *Nosema ceranae*. *Nosema apis* infections have been widely described (Hassanein, 1951; Wang and Moeller, 1969; Liu, 1984; Fries, 1988; de Graaf and Jacobs, 1991; Fries *et al*., 1992) and significantly, these have been reported from all continents where beekeeping involves the use of different subspecies and variants of *Apis mellifera*. It was not until recently, however, that infections due to *N. ceranae*, which seems to have jumped from its original host *A. cerana*, were described in *A. mellifera* (Higes *et al*., 2006; Huang *et al*., 2007). This microsporidian is currently considered as a major pathogen in European honey bees, and it has been shown to be pathogenic in this new host after experimental infection of worker caged bees (Higes *et al*., 2007, Paxton *et al*., 2007; Mayack and Naug, 2009). Indeed, natural infection has been associated with a syndrome of gradual depopulation (the unexplained disappearance of adult bees, a lack of attention to the brood, reduced colony vigour and heavy winter mortality), which is now a worldwide problem (Martín-Hernández *et al*., 2007; Higes *et al*., 2008 a,b; Plischuk *et al*., 2008). To date this widespread pathogen has been detected in four continents: Asia; Europe; North America; and South America (Martín-Hernández *et al*., 2007; Klee *et al*. 2007; Williams *et al*., 2008; Chen *et al*., 2008; Plischuck *et al*., 2008) but it has yet to be reported from an African country.

During February 2008, a total of 24 randomly selected samples of adult *Apis mellifera intermissa* worker honey bees (two samples of > 30 bees from each of 12 depopulated or asymptomatic colonies) were collected from an apiary suffering high colony losses with unexplained disappearance of adult forager bees in Sidi bel Abbes, Algeria (Fig. 1, 35º17'N, 2º57'W, 470 m above sea level). The presence of some dead bees close to the apiary and the absence of very slight dysentery were the only clinical data obtained from the beekeeper. When consulted, no other clinical signs had been observed in either adult bees or in the nest (no anatomic disturbances, no crawling or trembling bees, no dilated abdomens, etc).

The samples obtained were submitted to the Centro Apícola (CAR) laboratory (by Dr. Trouiller, Vita-Swarm) and analyzed using standard OIE methods (OIE, 2008) to detect the presence of *Nosema* spores, *Malphigamoeba* cysts, *Varroa destructor* and *Acarapis woodi*. The presence of *Nosema* spores was diagnosed by phase contrast microscopy and the corresponding species was confirmed by PCR using the 218MITOC and 321APIS primers for *N. apis* or *N. ceranae*, respectively, as described previously (Martín-Hernández *et al*., 2007). Each PCR was analyzed in a QIAxcel System (Qiagen), using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002). Negative controls (for extraction and PCR) were analysed in parallel to detect possible contamination. *Nosema* spp. were the only pathogens detected in the worker honey bees and while *N. ceranae* was present in eight of the 24 samples (positives samples from three depopulated and one asymptomatic colony), *N. apis* was found only in one asymptomatic sample.

This is the first time that *N. ceranae* has been detected in *Apis mellifera intermissa* colonies suffering from depopulation in the African continent, which provides evidence of a wider distribution of this emergent pathogen.

The recently demonstrated viability of spores inside the regurgitated pellets of the bee eater bird *Merops apiaster* has suggested an important role as fomites of infective spores of *Nosema* (Higes *et al.,* 2008a). The bee eater is a widely distributed and insectivorous, locally abundant mainly in arid and semi-arid areas of southern Europe, northern Africa and western Asia and it is strongly migratory, wintering usually in Africa. Birds can be seen in groups crossing the sea from Spain to North Africa usually in September,

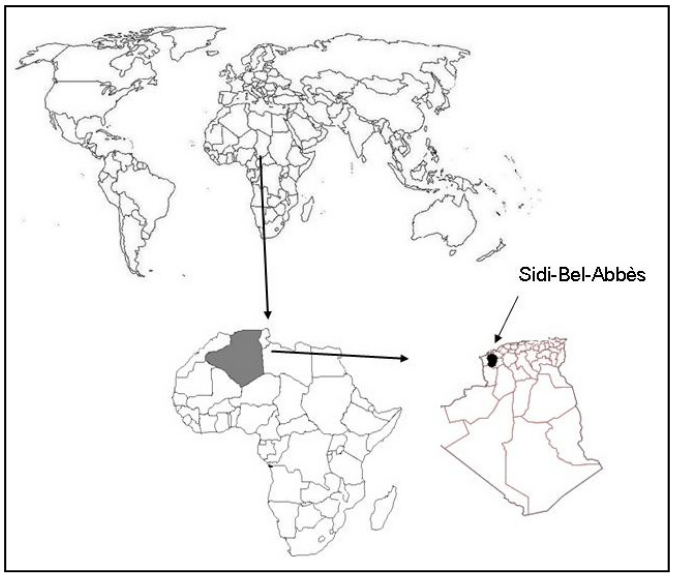


***Fig. 1.*** The sampling site at Sidi-Bel-Abbès, Algeria.

returning to Spain in April. The spreading of spores can easily be achieved during long migration journeys when birds have picked up spores by eating infected honey bee foragers. Apiaries are usually stop-over sites during migration, most of them critically located near fresh water and used year after year (Yosef *et al.*, 2006).

There are few data available from African countries regarding bee diseases and throughout the continent, *N. apis* has only been reported in *Apis mellifera scutellata* in the tropical climate of Zimbabwe (Fries *et al.*, 2003). Here, we have detected *N. ceranae* in depopulated colonies that display similar symptoms to those described in infected honey bee colonies in Europe and South America (Higes *et al.*, 2008b, 2009; Plischuck *et al.*, 2008; Van der Zee pers. comm., Borneck pers. comm.).

It was recently shown that *N. ceranae* infection can cause sudden collapse of bee colonies, establishing a direct correlation between *N. ceranae* infection and the depopulation of honey bee colonies under field conditions (Higes *et al.*, 2008b, 2009). The long asymptomatic incubation period would explain the failure to detect *N. ceranae* in many infected colonies (Martín-Hernández *et al.*, 2007). In general, African beekeepers do not consider classic nosemosis as an important disease, but perhaps this is due to the lack of reliable studies in this geographical area.

The climatic conditions in Sidi bel Abbas are similar to a dry Mediterranean climate, with little total annual rainfall (around 250-400 mm) mostly from October to March (Touazi *et al.*, 2004). These are like the conditions in Spain in 2004-2005, when *N. ceranae* was often detected and it was relevant in terms of its prevalence, always linked to clinical depopulation of honey bee colonies (Martín-Hernández *et al.*, 2007). The presence of this pathogen in Algeria confirms the plasticity of this microsporidian to diverse climatic conditions, showing a marked adaptation to warm and dry climates (Martín-Hernández *et al.*, 2009). It also emphasises the need for rigorous control in commercial trade to avoid the dispersion of this pathogen through the exchange of beekeeping material between countries. It is clear that epidemiological studies are necessary to increase our knowledge of the factors involved in the transmission and survival of this emergent pathogen. Studies must be carried out in more African countries in order to determine the dispersal of *N. ceranae* in this continent.

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# Artículo 6

## Efecto de la temperatura en el potencial biótico de los microsporidios que infectan a la abeja melífera

Raquel Martín-Hernández, Aránzazu Meana, Pilar García-Palencia, Pilar Marín, Cristina Botías, Encarna Garrido-Bailón, Laura Barrios y Mariano Higes

*Applied and Environmental Microbiology (2009) 75(8): 2554-2557*

*El ciclo biológico de* Nosema *spp. en las abejas depende de la temperatura. En este estudio, los potenciales bióticos de* Nosema apis *y de* Nosema ceranae *a 33°C resultaron ser similares, analizando éstos en función del número total de esporas contadas diariamente tras la infección. Sin embargo, en el caso de las abejas infectadas por* N. ceranae*, éstas mostraron una proporción mayor de fases inmaduras del microsporidio en sus células epiteliales del ventrículo en comparación con lo ocurrido en el caso de* N. apis.

*Por otro lado, a 25°C y a 37°C, el potencial biótico de* N. ceranae *fue mayor que el desarrollado por* N. apis.

*La mejor adaptación de* N. ceranae *para completar su ciclo endógeno a diferentes temperaturas claramente apoya la observación de los diferentes patrones epizootiológicos mostrados por ambos microsporidios.*

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# Effect of Temperature on the Biotic Potential of Honeybee Microsporidia▽

Raquel Martín-Hernández,[1] Aránzazu Meana,[2] Pilar García-Palencia,[2] Pilar Marín,[2] Cristina Botías,[1] Encarna Garrido-Bailón,[1] Laura Barrios,[3] and Mariano Higes[1]*

*Centro Apicola Regional, Bee Pathology Laboratory, Dirección General de Desarrollo Rural, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, 19180 Marchamalo, Spain[1]; Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain[2]; and Departamento de Estadística, CTI, Consejo Superior Investigaciones Científicas, 28006 Madrid, Spain[3]*

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**The biological cycle of *Nosema* spp. in honeybees depends on temperature. When expressed as total spore counts per day after infection, the biotic potentials of *Nosema apis* and *N. ceranae* at 33°C were similar, but a higher proportion of immature stages of *N. ceranae* than of *N. apis* were seen. At 25 and 37°C, the biotic potential of *N. ceranae* was higher than that of *N. apis*. The better adaptation of *N. ceranae* to complete its endogenous cycle at different temperatures clearly supports the observation of the different epidemiological patterns.**

Biotic potential represents the maximum reproductive capacity of a population under optimum environmental conditions. Thus, a species fulfilling its biotic potential would exhibit maximal exponential population growth, thereby augmenting the possibilities of transmission of the species. A wide range of factors affects the biotic potential of each species, and among the external factors, temperature clearly influences the life cycles of most parasitic species (4).

Nosemosis is a common worldwide disease of adult honeybees (*Apis mellifera*) that is caused by microsporidia (19). *Nosema apis* was the only agent known to produce this disease in *A. mellifera* until *N. ceranae* was identified in this host in 2005, in Europe (11) and Taiwan (12). Both these microsporidia infect and multiply in the ventricular cells of *A. mellifera*, and they can be found under different environmental conditions in both the northern and southern hemispheres (17, 13). Significantly, *N. ceranae* seems to be more pathogenic than *N. apis* in caged worker bees (10, 18), and it has recently been related to significant losses of bees and colony collapses under field conditions (17, 8).

Due to the lack of comparative studies of the factors affecting parasite virulence, trials were designed to determine the influence of temperature on the biotic potentials of both microsporidia. Only the deleterious effect of high exogenous temperature on spores of *N. apis* has been checked previously (16).

In this work, purified *N. apis* and *N. ceranae* spores with a minimum viability of 99% (tested with 4% trypan blue) were obtained from experimentally infected honeybees always maintained at 33°C as described previously (9). The spores were counted using a hemocytometer chamber (19), while the *Nosema* species identification was confirmed by PCR (17).

The experimental infection of bees was carried out as described previously (10). Briefly, young *Nosema*-free honeybees were starved for 2 h and fed 2 μl of 50% sucrose solution containing 100,000 viable *N. ceranae* or *N. apis* spores. Honeybees were anesthetized with $CO_2$, and later, a droplet of the spore solution was administered to each honeybee by touching a micropipette to its mouthparts until the entire droplet was consumed. The bees that did not consume the entire droplet were discarded. Uninfected control bees were fed 2 μl of 50% sucrose solution alone.

Three trials were carried out for 1 week each at three different temperatures (25, 33, and 37°C). Each trial included four replicate cages of 30 *N. apis*-infected honeybees, four replicate cages of 30 *N. ceranae*-infected honeybees, and four replicate cages of 30 uninfected honeybees. Three different refrigerated incubators (Memmert) were used for *N. ceranae*- or *N. apis*-infected bees and controls.

On days 1, 2, 3, 4, and 7 postinfection (p.i.), five living honeybees were removed from each of three cages ($n = 15$) in each incubator. Spore detection and counting were performed with each bee. To obtain the *Nosema* spore counts, the whole abdomen from each bee was placed into a sterile Eppendorf microtube in 200 μl of double-distilled water (PCR grade). After thorough grinding of the abdomen, the spore count for each bee was calculated with a hemocytometer. In addition, PCR was used to confirm the *Nosema* species identification. The biotic index was calculated as the total *N. apis* or *N. ceranae* spore count per day after infection.

Finally, on the same days p.i., a histopathological study was performed with two honeybees from the fourth cage in each incubator. The ventriculus of each bee, with the Malpighian tubules attached, was processed as described previously (10).

The daily spore count per bee and its relationship to the temperature were established with a nonparametric Mann-Whitney test for each *Nosema* species (level of significance, $\alpha = 0.05$). Two curvilinear regression models for each temperature were established. The growth curves fitted predict the increases of the *N. ceranae* and *N. apis* spore counts over time (days p.i.), and the slopes and intercepts of the regression lines could be compared. SPSS (version 15.0) software was used to perform the tests and regression analysis.

* Corresponding author. Mailing address: Centro Apícola Regional, Junta de Comunidades de Castilla-La Mancha, 19180 Marchamalo, Spain. Phone: 34 949 250 026. Fax: 34 949 250 176. E-mail: mhiges@jccm.es.

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TABLE 1. Percentages of infected honey bees in the different test groups[a]

| Group | Temp (°C) | % of infected bees on p.i. day: | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 7 |
| *N. apis*-exposed bees | 25 | 33 | 53 | 100 | 100 | 100 |
| | 33 | 66 | 93 | 100 | 100 | 100 |
| | 37 | 53 | 60 | 60 | 60 | 0 |
| *N. ceranae*-exposed bees | 25 | 100 | 100 | 100 | 100 | 100 |
| | 33 | 100 | 100 | 100 | 100 | 100 |
| | 37 | 100 | 100 | 100 | 100 | 100 |
| Controls | 25 | 0 | 0 | 0 | 0 | 0 |
| | 33 | 0 | 0 | 0 | 0 | 0 |
| | 37 | 0 | 0 | 0 | 0 | 0 |

[a] A bee is considered to be infected when at least one spore is observed in the hemocytometer's entire central square millimeter grid (19).

Throughout the study, *Nosema* infection was not evident in any of the control bees (Table 1), while *N. ceranae* was detected in all the bees (100%) exposed to this microsporidium. The proportion of the bees exposed to *N. apis* in which *N. apis* infection was detected varied during the period studied (0 to 100%).

The biotic indices for the two microsporidia at 33°C were similar ($P > 0.05$; Mann-Whitney U test), and the lowest biotic index was recorded for microsporidia kept at 37°C. The indices for *N. ceranae* were higher than those for *N. apis* at 25 and 37°C ($P < 0.05$; Mann-Whitney U test), and even the *N. ceranae* spore counts were much higher than the *N. apis* spore counts at 33°C during the first 3 days after infection ($P < 0.05$; Mann-Whitney U test). However, this difference between the microsporidia incubated at 33°C was no longer evident by the end of the study period ($P > 0.05$; Mann-Whitney U test). Nevertheless, *N. ceranae* adapted to the temperature better than *N. apis* when the cages were incubated at 25°C, with a spore count more than double that for *N. apis* on each day of sampling ($P < 0.05$; Mann-Whitney U test). The spore counts for both microsporidia at 37°C were always lowest, and the spore count for *N. ceranae* was always higher than that for *N. apis* at this temperature ($P < 0.05$; Mann-Whitney U test).

The increase in the biotic index of *N. ceranae* at different temperatures fits well ($R^2 > 0.7$) with a predictable model (Fig. 1). The biotic index at 33°C was predictably higher than that at any other temperature studied, as the slope (0.78) reflects a higher number of spores at this temperature than at any of the others. In contrast, the biotic index of *N. apis* does not fit with any model at any temperature ($R^2$, 0.554 for 33°C). The values obtained for *N. apis* spores were always lower than those predicted.

Cells parasitized by *N. apis* and *N. ceranae* were observed only in the ventricular epithelium and never in Malpighian tubules or muscular layers.

The two microsporidia produced similar morphological changes in the epithelial ventricular cells, as described previously (10), although there was variability in the number of cells

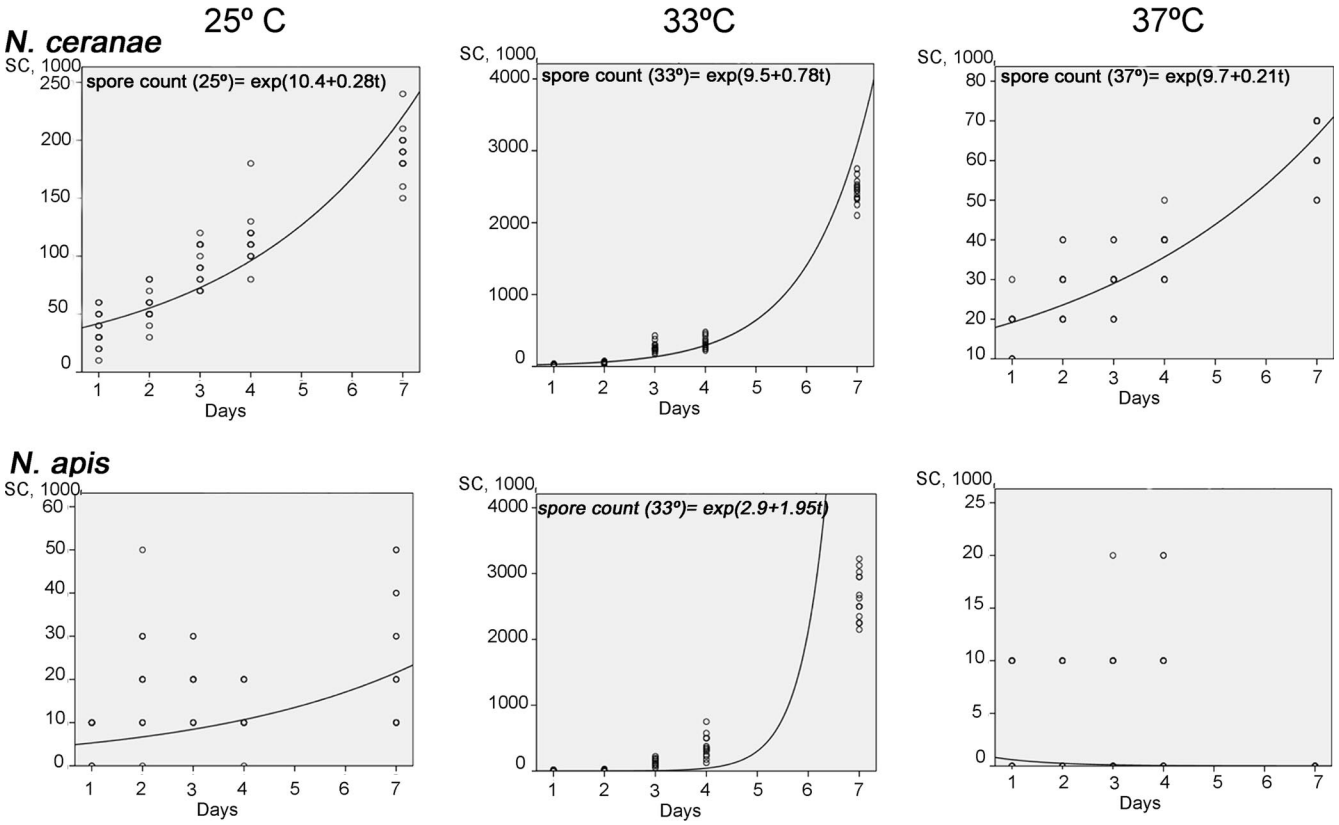


FIG. 1. Growth curves. Dependent variable: increasing of *N. ceranae* and *N. apis* spore counts (SC). Independent variable: days p.i.

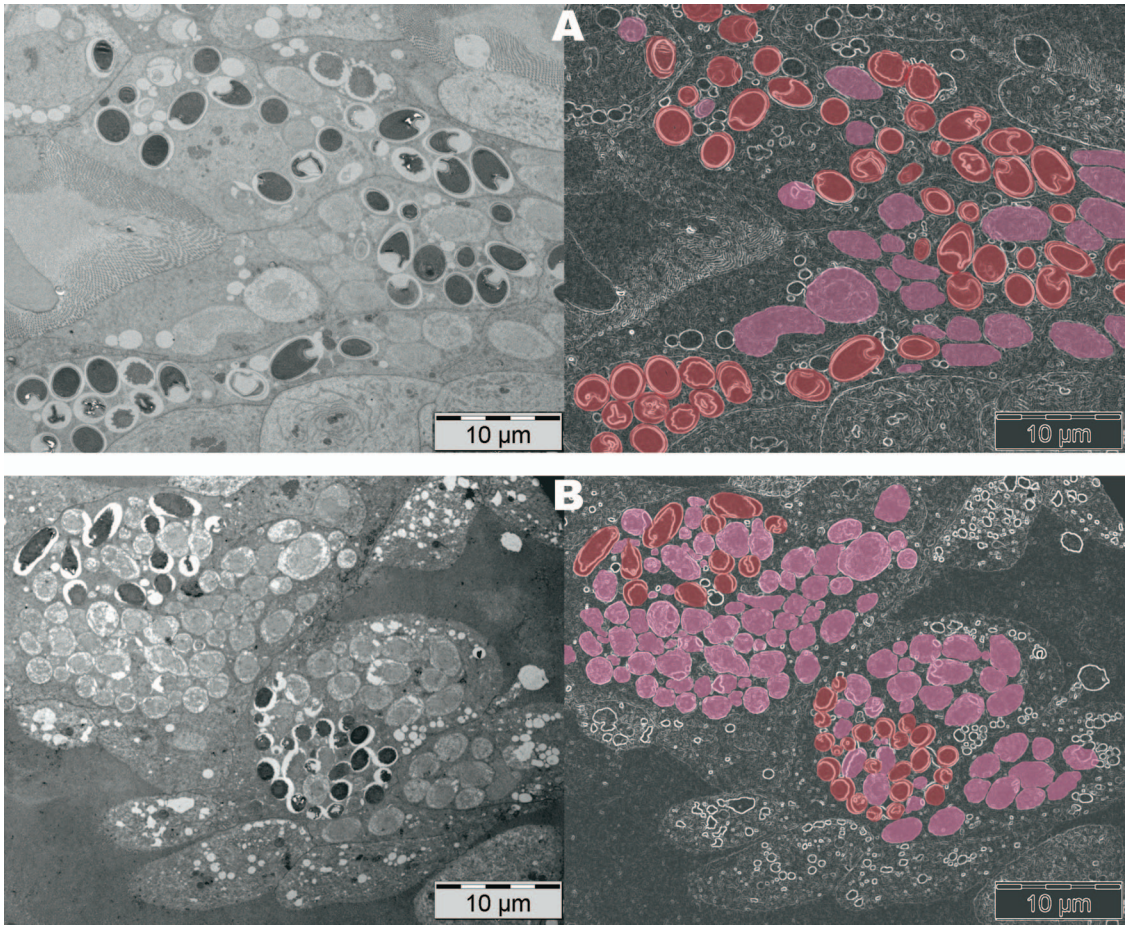


FIG. 2. Detailed views of ventricular epithelial cells parasitized at 7 days p.i. at 33°C. *N. apis*-infected cells (A) displayed similar quantities of immature and mature stages (red), while *N. ceranae*-infected cells (B) exhibited a higher proportion of immature stages (pink) at this time.

infected and in the development of the infection, depending on the temperature. At 33°C, both microsporidia developed very well and parasitized cells could be seen from day 2 p.i. Indeed, empty spores were seen in cells parasitized by either microsporidium, confirming the completion of the endogenous cycle in less than 2 days. During the initial days, no differences in the calculated parasitic infection ratios were detected, although *N. ceranae* infection affected more cells than *N. apis* infection on day 4 p.i. (mean ± standard deviation, 76.14% ± 15.5% versus 54.6% ± 14.6%) and day 7 p.i. (83.14% ± 10.7% versus 80.3% ± 12.8%) and there was evidence of a higher proportion of immature stages of *N. ceranae* (70%) than of mature stages (Fig. 2B). In contrast, *N. apis*-infected cells displayed similar quantities of immature and mature stages (50% each) at this time (Fig. 2A). This finding may explain why the pathological consequences of infections with the two parasites are not the same, even when similar spore counts are detected. *N. ceranae* infected more epithelial cells than *N. apis*, but due to the impossibility of counting immature forms in a hemocytometer, the real number of affected cells in the bees cannot be accurately evaluated by using total spore counts. This approach is unreliable as a tool to evaluate the health status of infected bees, as also shown recently in field trials (8). Thus, a more reliable procedure must be used to include immature stages in these measurements. Since transmission electron microscopy evaluation is expensive and time-consuming and cannot be considered for routine analysis, quantitative real-time PCR may be a useful tool in this sense.

*N. ceranae* infection affects more cells than *N. apis* infection when honeybees are maintained at the same temperature, and such differences may explain the higher mortality observed when *N. ceranae* infects *A. mellifera* than when *N. apis* is the infecting agent (9, 18).

*N. ceranae* clearly infected bees kept at 25°C by day 2, when infected cells were easily seen in all the visualized fields, while *N. apis*-infected cells were not seen until day 7, when at least one parasitized cell could be detected in almost all areas (Fig. 3).

The temperature of 37°C was the least favorable for either species, as very few parasitized cells were detected in *N. ceranae*-infected bees and none were detected in *N. apis*-infected bees at this temperature.

Nevertheless, the fact that a few infected cells could be found in all the areas studied from day 2 onwards indicated that *N. ceranae* was at least capable of infecting cells at this temperature. Neither spores nor infected cells in *N. apis*-infected bees kept at 37°C were detected on day 7 p.i., confirming the data from previous studies with this microsporidium spe-

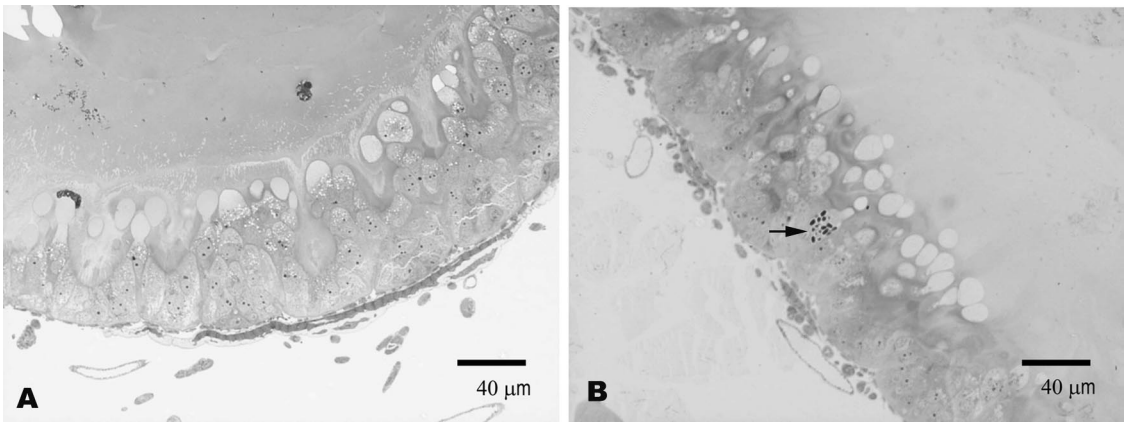


FIG. 3. Ventricular epithelial cells 2 days p.i. at 25°C. In honeybees infected by *N. apis*, no parasitic forms were detected (A), but different parasitic stages (arrow) in cells from *N. ceranae*-infected honeybees were seen (B).

cies (3). Indeed, a previous study reported the temperature of 37°C to be the maximal growth temperature for *N. apis* (15) and also stated that *Nosema*-infected bees recover when kept at this temperature. *Nosema* spores counted at 37°C in the previous days may be remanent spores in transit.

The better adaptation of *N. ceranae* than of *N. apis* to temperature, enabling it to complete its endogenous cycle with a higher biotic index, is clearly in agreement with the epidemiological differences between these microsporidia. Indeed, while *N. ceranae*-infected honeybees can be detected in all four seasons (17, 8), *N. apis* infection is more prevalent in milder seasons such as the spring and autumn (1, 6, 7, 19).

It is generally accepted that the earth's temperature is progressively increasing, and the consequences of this effect on the endogenous and external life cycles of parasites are of concern (2). As described previously for *Aethina tumida* (14), increasing temperatures due to climate change will promote the extension of the distribution of honeybee pathogens or pests. Changes in climate may affect the distribution, seasonality, and severity of infectious diseases (5), such as nosemosis in honeybees, and the plasticities of species to adapt to new triggering situations will increase the probabilities not only of colonizing but also of consolidating the occupancy of new ecosystems under different environmental conditions.

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# Artículo 7

## Desarrollo diferencial de los microsporidios que infectan a las abejas melíferas al aplicar temperaturas de incubación crecientes

Mariano Higes, Pilar García-Palencia, Cristina Botías, Aránzazu Meana y Raquel Martín-Hernández

*Environmental Microbiology Reports (2010) 2(6): 745-748*

*En el último siglo, la nosemosis causada por* N. apis *ha sido generalmente considerada como una enfermedad de baja prevalencia presentada por* Apis mellifera*, a pesar de encontrarse distribuida por todo el mundo. Las colonias infectadas por* N. apis *desarrollan bajos niveles de infección durante el verano, un pequeño pico en otoño y normalmente un pequeño incremento durante el invierno. Sin embargo, la nosemosis debida a* Nosema ceranae *se considera como una enfermedad emergente que está suponiendo una seria amenaza para la salud de las abejas a nivel individual, así como para la colonia.*

*Los signos clínicos de la infección por parte de estos dos patógenos son muy diferentes, así como su virulencia, su distribución y patogenicidad. En este estudio se ha llevado a cabo un experimento en el cual abejas obreras infectadas de manera artificial fueron mantenidas en condiciones de laboratorio a dos temperaturas diferentes. Ambos microsporidios se desarrollaron según lo esperado después de 4 días post-infección a 33$^{o}$C, pero cuando estas abejas infectadas fueron mantenidas durante 5 a 7 días a 37,2$^{o}$C, únicamente* N. ceranae *fue capaz de completar su ciclo biológico en los ventrículos de las abejas infectadas, mientras que el desarrollo de* N. apis *se vio inhibido. Estos resultados, junto con otros anteriormente mostrados, sugerirían que* N. ceranae *podría tratarse de una especie euriterma, mientras que* N. apis *podría ser una especie estenoterma. La mayor tolerancia a la temperatura mostrada por* N. ceranae *podría estar relacionada con su mayor prevalencia a nivel mundial.*

***PRESENTACIÓN EN CONGRESO:***

*Higes M, Martín-Hernández R, Botías C, García-Palencia P, Marín P, Meana A. Efecto de la elevación térmica en el ciclo biológico endógeno de microsporidios que infectan* Apis mellifera*. IX Congreso Ibérico de Parasitología, 15-18 de Septiembre 2009, Lisboa (Portugal).*

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Environmental Microbiology Reports (2010) **2**(6), 745–748 doi:10.1111/j.1758-2229.2010.00170.x

# The differential development of microsporidia infecting worker honey bee (*Apis mellifera*) at increasing incubation temperature

**Mariano Higes,[1]* Pilar García-Palencia,[2] Cristina Botías,[1] Aránzazu Meana[2] and Raquel Martín-Hernández[1]**

[1]*Centro Apícola Regional, Bee Pathology Laboratory, Consejería de Agricultura, Junta de Comunidades de Castilla – La Mancha, 19180 Marchamalo, Spain.*

[2]*Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.*

## Summary

**In the last century, nosemosis caused by *Nosema apis* is traditionally considered as a low-prevalence disease of *Apis mellifera*, even though it occurs worldwide. Colonies affected by *N. apis* display low levels of infection during summer, a small peak in autumn and usually a slow rise during winter. However, nosemosis due to *Nosema ceranae* is considered as an emergent illness that is posing a major threat to the health of individual honey bees and whole bee colonies. The symptoms of infection by these two pathogens are very different, as are the virulence, spread and pathogenicity. We have carried out experiments in artificially infected worker honey bees maintained in the laboratory at two different temperatures. Both microsporidia developed as expected for up to 4 days after infection at 33.0°C, but when maintained for 5 or 7 days at 37.2°C, only *N. ceranae* completed its life cycle in infected honey bees, while the development of *N. apis* was inhibited. This and other published data suggest that *N. ceranae* is eurythermal whereas *N. apis* is stenothermal. The higher temperature tolerance recorded may be related to the higher prevalence of *N. ceranae* reported worldwide.**

## Introduction

*Nosema apis* causes one of the most important diseases of honey bees, although it is generally overlooked by beekeepers (Burnside and Revell, 1948; Hornitzky, 2005). Outbreaks of nosemosis were believed to be the result of poor beekeeping practices or other problems, such as poor wintering conditions, poor dietary pollen quality or other pathologies or toxins. Infection by *N. apis* gradually declines during the warm summer months until it disappears, although it generally resurges later in autumn and spring (Doull and Eckert, 1962). These epidemiological cycles may reflect the favourable environmental temperatures in these seasons (Burnside and Revell, 1948; Dyes and Wilson, 1978) and the decrease in viable spores inside the hive (Bailey, 1955). Indeed, in honey bees, *N. apis* spores are rarely found in summer or winter, and they may only be detected in heavily infected honey bee colonies (OIE, 2008).

In contrast, emergent nosemosis caused by *Nosema ceranae*, now named nosemosis type C (COLOSS Workshop, 2009; Higes *et al*., 2010), is considered a major threat to honey bee colonies (Higes *et al*., 2008a; Chen *et al*., 2009; Cornman *et al*., 2009) and infection produces symptoms very different from *N. apis* (Higes *et al*., 2008a; 2009; 2010; COLOSS Workshop, 2009). In recent years, natural infection by this microsporidian has been associated with a gradual loss of worker and forager bees, copious colony death in the autumn or winter, and poor honey production (Higes *et al*., 2006; 2008a; 2009; Martín-Hernández *et al*., 2007; Botías *et al*., 2009; Korpela, 2009). In addition, infection by *N. ceranae* suppresses the honey bee's immune response and induces senescence (Antúnez *et al*., 2009), and it now seems to be capable of infecting other hymenoptera (Plischuk *et al*., 2009), properties not yet associated with *N. apis*.

Temperature clearly affects the endogenous life cycle of both microsporidia distinctly (Martín-Hernández *et al*., 2009) and although the biotic potential was similar for both *N. apis* and *N. ceranae* at 33.0°C, this index was higher at 25.0°C and 37.0°C for *N. ceranae*. The better capacity of *N. ceranae* to adapt to and complete its endogenous cycle at different temperatures reflects the epidemiological differences between these microsporidia in field conditions.

The aim of this study was to examine the higher tolerance of *N. ceranae* to high temperatures when compared with *N. apis*. Accordingly, experimentally infected worker honey bees were maintained at 33.0°C to enhance their

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endogenous life cycle (as described Martín-Hernández *et al*., 2009) and then the temperature was shifted to 37.2°C, which has been reported to inhibit *N. apis* development (Burnside and Revell, 1948).

## Results and discussion

At 33.0°C, both microsporidia developed as expected from day 4 pi (post infection: Martín-Hernández *et al*., 2009), as evident from the intracellular stages of each microsporidium in the ventricular epithelial cells of *N. apis*- and *N. ceranae*-infected worker honey bees (Fig. 1A and B). In contrast, no *Nosema* infection was evident in any of the control uninfected bees throughout the study [transmission electron microscopy (TEM) studies and mean spore count]. When the infected worker honey bees were shifted to 37.2°C for 5 days, no immature stages of the microsporidium were observed in the ventricular cells of bees infected with *N. apis*. Indeed, the mature spores of this species displayed clear signs of degeneration, such as the absence of polar filament coils, a nucleus and other such structures (Fig. 2A). In contrast, not only were some immature stages found inside the ventricular epithelial cells in the worker honey bees infected by *N. ceranae* but also, infective mature spores with structures characteristic of this microsporidium were evident, indicating that *N. ceranae* were still multiplying (Fig. 2B).

When these bees were maintained for 2 more days at 37.2°C (day 11 pi), mature and immature stages of the microsporidia were only visible inside the ventricular epithelial cells of bees infected by *N. ceranae*. In bees infected by *N. apis* there was no evidence of this microporidia at any stages of its life cycle in the ventricular epithelial cells.

On day 12 pi, all honey bees infected by *N. ceranae* were dead ($n = 9$ for each replicate) with a mean mature spore count per bee of 421 ($\pm$ 21.1) $\times 10^3$ (mean $\pm$ standard deviation). In contrast, bees in the control group

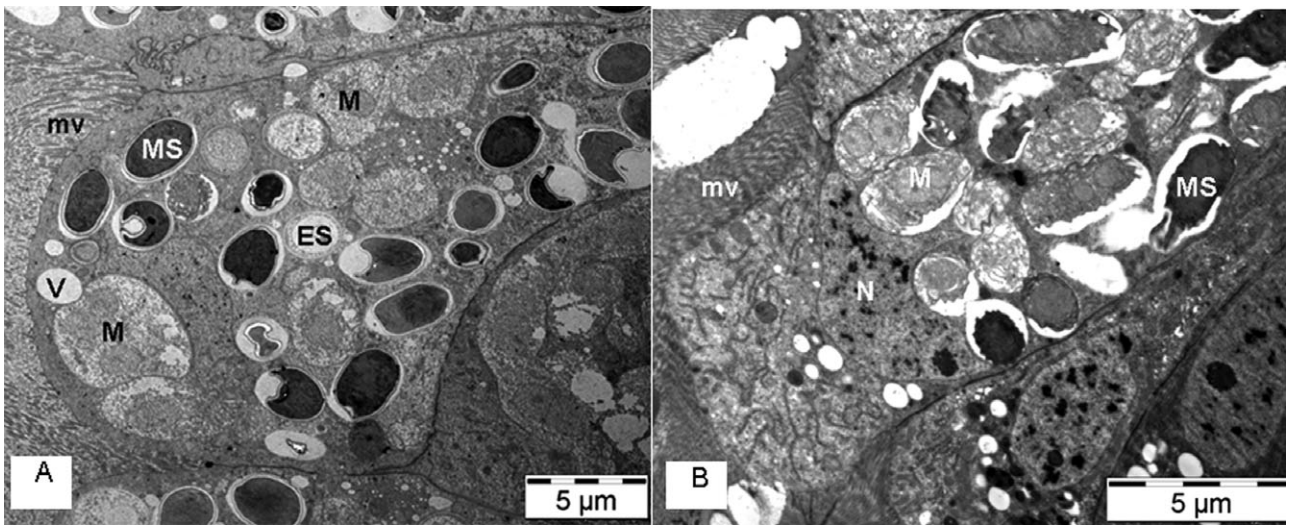


**Fig. 1.** A frame with capped brood of *Apis mellifera* worker bees was taken from a *Nosema*-free colony and maintained in an incubator at 35°C until the bees were born (Higes *et al*., 2007; Martín-Hernández *et al*., 2009). Newly emerged worker bees were carefully removed from the frame, confined to cages and kept in a different incubator for 5 days at 33°C. On the day of infection, 10 bees from each cage were taken to confirm the absence of *Nosema* spp. by PCR, and they were infected as described in Higes and colleagues (2007). Briefly, 5-day-old bees were starved for 2 h and subsequently, each bee was fed with 2 µl of water containing 100 000 viable *Nosema ceranae* or *Nosema apis* spores while uninfected control bees were fed with 2 µl of water alone. Infections were carried out with purified *N. apis* and *N. ceranae* spores (confirmed by PCR; Martín-Hernández *et al*., 2007) with a minimum viability of 99% (as tested with 0.4% trypan blue: Higes *et al*., 2008b; Martín-Hernández *et al*., 2009). The experiment involved the use of two groups of 15 *N. apis*-infected honey bees, two groups of 15 *N. ceranae*-infected honey bees, and two groups of 15 uninfected honey bees (control). Three different incubators (33°C) were used for the *N. apis*- and *N. ceranae*-infected or the controls bees (Martín-Hernández *et al*., 2009). Four days post infection (pi), two living honey bees were removed from each of the replicate groups ($n = 12$ bees) and their ventriculi, with the Malpighian tubes attached, were processed as described previously (Higes *et al*., 2007; 2008a; 2009) for transmission electron microscopy (TEM) studies to confirm the successful infection. At that time (4 day pi), the temperature was increased in the three incubators to 37.2°C and the living bees were maintained at this temperature for up to 11 days pi in new cages (to avoid them contacting the spores that were contaminating them). Subsequently, two honey bees from each replicate group were processed for TEM on days 9 and 11 pi as indicated previously. The midguts from the bees remaining at 11 pi ($n = 9$ for each replicate) were individually placed in a sterile microtube in 200 µl of $ddH_2O$ (PCR grade). After grinding thoroughly (Eppendorf), the spore count for each bee was calculated with a haemocytometer and in addition, PCR was used to confirm the *Nosema* species present.
A. Ventricular epithelial cell infected by *N. apis* from 4 days pi at 33°C filled with different parasitic stages: meronts (M), mature spores (MS), empty spore (ES), Vacuoles (V), Microvilli (mv). Scale bar = 5 µm.
B. Ventricular epithelial cell infected by *N. ceranae* from 4 days pi at 33°C. Different parasitic stages: meronts (M), mature spores (MS). Note the apically displaced nucleus (N). Scale bar = 5 µm.

 

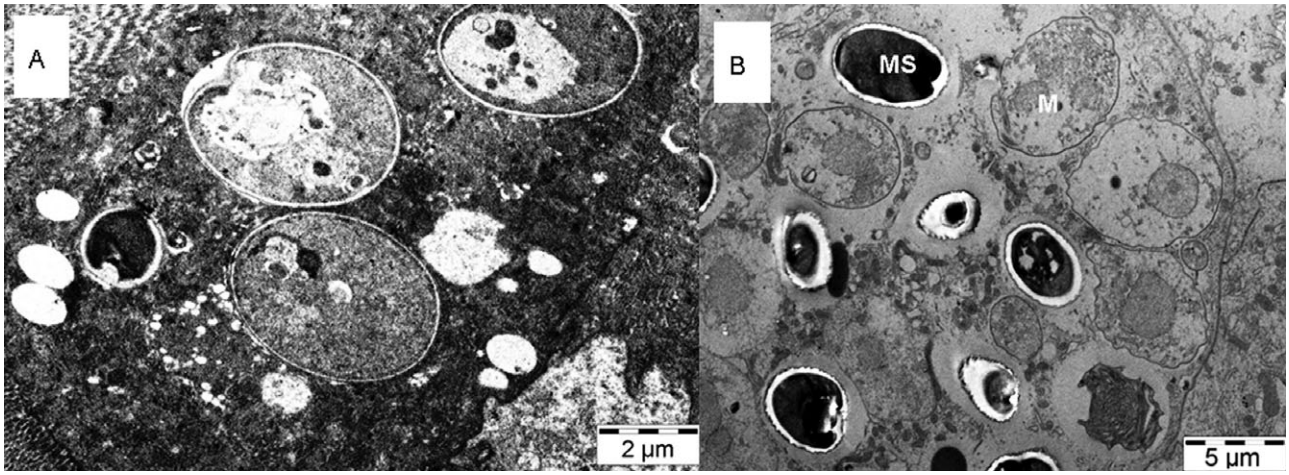


**Fig. 2.** A. Ventricular epithelial cell infected by *Nosema apis* after 5 days at 37.2°C. Degenerated mature spores and numerous vacuoles in the cytoplasm. Scale bar = 2 µm.
B. Ventricular epithelial cell infected by *Nosema ceranae* after 5 days at 37.2°C. Different parasitic stages: meronts (M), mature spores (MS). Moderate cell degeneration. Scale bar = 5 µm.

or those infected with *N. apis* remained alive ($n = 9$ for each group) and no spores were detected.

We have shown that *N. ceranae* is capable of completing its full biological life cycle at temperatures as high as 37.2°C, temperatures that appear to inhibit the development of *N. apis* in TEM studies and through the mean spore count. These results agree with previous data demonstrating that honey bees infected by *N. apis* recover when they are kept at 37.0–37.2°C for 1–14 days (Karmo and Morgenthaler, 1939; Lotmar, 1944; Burnside and Revell, 1948) and that the viability of *N. apis* spores decreases steadily over time at temperatures close to 40°C (Malone *et al*., 2001). In the honey bees infected with *N. apis*, mature spores within the epithelial ventricular cells display clear signs of degeneration when they were maintained for 5 days at 37.2°C. Indeed, at this temperature all the parasitic forms of this microsporidium had disappeared 2 days later and no mature spores were detected in the bees. In contrast, the full biological life cycle was still completed in the bees infected with *N. ceranae*, and mature and immature stages of this microporidium could be observed inside the epithelial ventricular cells at this temperature, finally leading to the death of the infected workers. Indeed, in terms of survival, these results are in agreement with previous studies suggesting that *N. ceranae* is more virulent than *N. apis* in caged worker honey bees (Higes *et al*., 2007; Paxton *et al*., 2007).

In addition, these results confirmed earlier data demonstrating that *N. ceranae* is better adapted to different temperatures (Martín-Hernández *et al*., 2009), and that it can complete its endogenous cycle with a higher biotic index from 25.0°C to 37.0°C, supporting the epidemiological differences between these microsporidia in field conditions. Indeed, while mature and immature stages of *N. ceranae* can be detected in infected honey bees in all four seasons (Martín-Hernández *et al*., 2007; Higes *et al*., 2008a), *N. apis* infection is more prevalent in the milder spring or autumn seasons (Bailey, 1955; Doull and Eckert, 1962; Dyes and Wilson, 1978; OIE, 2008) when temperatures are less extreme than in winter or summer.

The presence of *N. ceranae* in colder, and especially in hotter months, may be due to its eurythermality (Martín-Hernández *et al*., 2009), a characteristic that could contribute to the higher worldwide prevalence of *N. ceranae*, and to its importance in causing an emerging worldwide disease named type C nosemosis (COLOSS Workshop, 2009; Higes *et al*., 2010). Global climate change produces ecological perturbations that cause geographical and phenological shifts, as well as altering the dynamics of parasite transmission. Indeed, on this basis climate change appears to enhance host-switching (Brooks and Hoberg, 2007) and it may also affect the conditions of certain microhabitats, facilitating the colonization of novel areas by parasites.

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# Artículo 8

## Egagrópilas de *Merops apiaster* como fómites de esporas infectivas de *Nosema ceranae*

Mariano Higes, Raquel Martín-Hernández. Encarna Garrido-Bailón, Cristina Botías, Pilar García-Palencia y Aránzazu Meana

*Environmental Microbiology (2008) 10(5): 1374-1379*

*En el último siglo, la nosemosis causada por* Nosema apis *ha sido generalmente considerada como una enfermedad de baja prevalencia presentada por* Apis mellifera*, a pesar de encontrarse distribuida por todo el mundo. Las colonias infectadas por* N. apis *desarrollan bajos niveles de infección durante el verano, un pequeño pico en otoño y normalmente un pequeño incremento durante el invierno. Sin embargo, la nosemosis debida a* Nosema ceranae *se considera como una enfermedad emergente que está suponiendo una seria amenaza para la salud de las abejas a nivel individual, así como para la colonia.*

*Los signos clínicos de la infección por parte de estos dos patógenos son muy diferentes, así como su virulencia, su distribución y patogenicidad. En este estudio se ha llevado a cabo un experimento en el cual abejas obreras infectadas de manera artificial fueron mantenidas en condiciones de laboratorio a dos temperaturas diferentes. Ambos microsporidios se desarrollaron según lo esperado después de 4 días post-infección a 33 $^{o}$C, pero cuando estas abejas infectadas fueron mantenidas durante 5 a 7 días a 37.2 $^{o}$C, únicamente* N. ceranae *fue capaz de completar su ciclo biológico en los ventrículos de las abejas infectadas, mientras que el desarrollo de* N. apis *se vio inhibido. Estos resultados, junto con otros anteriormente mostrados, sugerirían que* N. ceranae *podría tratarse de una especie euriterma, mientras que* N. apis *podría ser una especie estenoterma. La mayor tolerancia a la temperatura mostrada por* N. ceranae *podría estar relacionada con su mayor prevalencia a nivel mundial.*

***PRESENTACIÓN EN CONGRESO:***

*Higes M, Martín-Hernández R, Botías C, García-Palencia P, Marín P, Meana A. Efecto de la elevación térmica en el ciclo biológico endógeno de microsporidios que infectan* Apis mellifera*. IX Congreso Ibérico de Parasitología, 15-18 de Septiembre 2009, Lisboa (Portugal).*

Environmental Microbiology (2008) **10**(5), 1374–1379 doi:10.1111/j.1462-2920.2007.01548.x

Brief report

# Regurgitated pellets of *Merops apiaster* as fomites of infective *Nosema ceranae* (Microsporidia) spores

**Mariano Higes,[1]* Raquel Martín-Hernández,[1] Encarna Garrido-Bailón,[1] Cristina Botías,[1] Pilar García-Palencia[2] and Aránzazu Meana[3]**
[1]*Regional Apicultural Center, Dirección General de la Producción Agropecuaria, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, Marchamalo, Guadalajara, Spain.*
[2]*Department of Veterinary Medicine and Surgery, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, Spain.*
[3]*Department of Animal Health, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, Spain.*

**Summary**

**The importance of transmission factor identification is of great epidemiological significance. The bee-eater (*Merops apiaster*) is a widely distributed insectivorous bird, locally abundant mainly in arid and semi-arid areas of southern Europe, northern Africa and western Asia but recently has been seen breeding in central Europe and Great Britain. Bee-eaters predominantly eat insects, especially bees, wasps and hornets. On the other hand, *Nosema ceranae* is a Microsporidia recently described as a parasite in *Apis mellifera* honeybees in Europe. Due to the short time since its description scarce epidemiological data are available. In this study we investigate the role of the regurgitated pellets of the European bee-eater as fomites of infective spores of *N. ceranae.* Spore detection in regurgitated pellets of *M. apiaster* is described [phase-contrast microscopy (PCM) and polymerase chain reaction (PCR) methods]. Eighteen days after collection *N. ceranae* spores still remain viable and their infectivity is shown after artificial infection of *Nosema*-free 8-day-old adult bees. The epidemiological consequences of the presence of *Nosema* spores in this fomites are discussed.**

Received 20 July, 2007; accepted 27 November, 2007. *For correspondence. E-mail mhiges@jccm.es; Tel. (+34) 949250 026; Fax (+34) 949250 176.

## Introduction

The importance of transmission factors identification is basic from an epidemiological perspective as it allows a better understanding of the spread of the disease and contributes to an efficient disease management. Several inanimate objects have been identified as fomites because they harbour a pathogenic organism and facilitate their spreading. Dispersal of pathogens by fomites can contribute to new spatiotemporal infection patterns (Gustafson *et al*., 2007). Here we report the role of an insectivorous bird species, the European bee-eater, as a spreader of a Microsporidia, a pathogen present in its regurgitated pellet.

The European bee-eater, *Merops apiaster* (Fig. 1), is a widely distributed insectivorous migrant bird of the *Meropidae* family. It is locally abundant mainly in arid and semi-arid areas (Cramp, 1985). It breeds in southern Europe and in parts of northern Africa and western Asia, but recently it has been seen breeding in central Europe and Great Britain (Fraser and Rogers, 2001). As the name suggests, bee-eaters feed mainly on insects, especially bees, wasps and hornets, which are caught in the air by sorties from an open perch. Several studies have shown that Hymenoptera (mostly *Apis mellifera*) is the most important prey item in the diet of the European bee-eater (revised by Cramp, 1985) and in Spain it ranges from 69.4% to 82% (Martínez, 1984) of its hole diet. After ingestion, the indigestible part of insects is compressed into a pellet by the grizzard and then this pellet will be eventually regurgitated. These regurgitated pellets are composed mainly of exoskeleton of numerous insect individuals and are usually elongated dark structures with an average size of 18–25 × 8–15 mm.

Microsporidia are intracellular obligate parasites ubiquitous in nature, infecting all animal phyla (Canning and Lom, 1986; Weiss, 2003). In the last few years, *Nosema ceranae* (Table 1, Fig. 2F) has been described as a parasite of the *A. mellifera* honeybee (Higes *et al*., 2006) widely distributed (Huang *et al*., 2007; Klee *et al*., 2007; Martín-Hernández *et al*., 2007). This microsporidia has shown to be highly pathogenic for this new host (Higes *et al*., 2007a), although due to its very recent description,


**Fig. 1.** Bee-eater (*Merops apiaster*).

scarce epidemiological data are available. Data related with spore sources for bees and modes of infection transmission are urgently needed.

In this study, viable spore detection in regurgitated pellets of *M. apiaster* is described, and spore infectivity after artificial infection of *Nosema*-free adult bees is demonstrated.

## Results and discussion

This is the first report on Microsporidia infective spores detection in regurgitated pellets of *M. apiaster*. Recently, corbicular pollen has been reported as a possible *N. ceranae* reservoir (Higes *et al*., 2007b). The presence of *N. ceranae* spores in the bee-eater's regurgitated pellets is a new finding with potential epidemiological repercussions.

*Nosema* spores were confirmed in all the samples of the regurgitated pellets for 2006 and 2007, both by phase-contrast microscopy (PCM) and by polymerase chain reaction (PCR), and the species was confirmed as *N. ceranae*. However, the faeces samples came to be always negative by both methods. Human-associated microsporidia *Encephalitozoon intestinalis*, *Enterocytozoon bieneusi* and *Enterocytozoon hellem* have been detected in urban park pigeons faecal samples (Haro *et al*., 2005). The absence of *Nosema* spores in the faeces of *M. apiaster* can only be explained due to the low number of studied samples or because the digestive process of the bird could be able to damage the spores.

Experimentally infected bees tested positive for PCM spore detection both 3 days post infection (PI) and 7 days p.i. Control bees tested negative during every analyses, and all but one were alive at day 21 p.i. Quantification of spores showed a 10-fold increase in the number of spores, from 100 000 on day 3 to one million on day 7. Twenty days p.i. the infected bees showed symptoms similar to those previously described (Higes *et al*., 2007a) when they became visibly less active. On day 21 p.i. all the infected bees remaining were dead. Most of the epithelial cells were plenty of spores and immature. Scarce unaltered cells were also observed, but either at the tip or at the bottom of epithelium folds contained *N. ceranae* intracellular stages (Fig. 2E and F). Spore quantification of dead infected bees showed a mean of 22.5 million spores per bee in cage 1 and 25 million spores per bee in replicated cage 2. No signs of dysentery were seen throughout the study.

Viability of spores was always very high in the regurgitated pellets (more than 80%) and in the control tube (100%) and no statistically differences were found in viability percentage throughout the time (ANOVA, $P > 0.05$). A decrease in the parasitic load was also registered in all analysed samples. As that progressive decrease in the number of spores was observed in control tubes as well as in pellets, this seems to indicate that this loss happens under natural conditions and that the pellets do not provide protection to the spores (Table 2). However, despite this diminution in the parasitic load the remaining spores are still infective as is demonstrated by the infection of naïve bees with spores that had been exposed to normal climatic conditions for 18 days (Fig. 3).

An analysis of the diet of the European bee-eater has shown that it is mostly made up of Hymenoptera with a high

**Table 1.** *Nosema ceranae* biological characteristics

| | |
|---|---|
| Taxonomy (Adl *et al*., 2005) | Eukaryota; Opistokonta; Fungi; Microsporidia; *Nosema* |
| Spores (Fries *et al*., 1996) | Ovocylindrical: 4.7 × 2.7 μm (fresh) to 3.4 × 1.7 (fixed and stained) |
| | Polar filament with 20–23 coils |
| Cycle features in *Apis mellifera* (Higes *et al*., 2007a; Meana *et al*., 2007) | Endogenous life cycle: less than 3 days |
| | Disporoblastic |
| | Diplokariotics |
| | All parasitic stages in direct contact with the host cell cytoplasm |
| | Intracellular germination of spores |

 

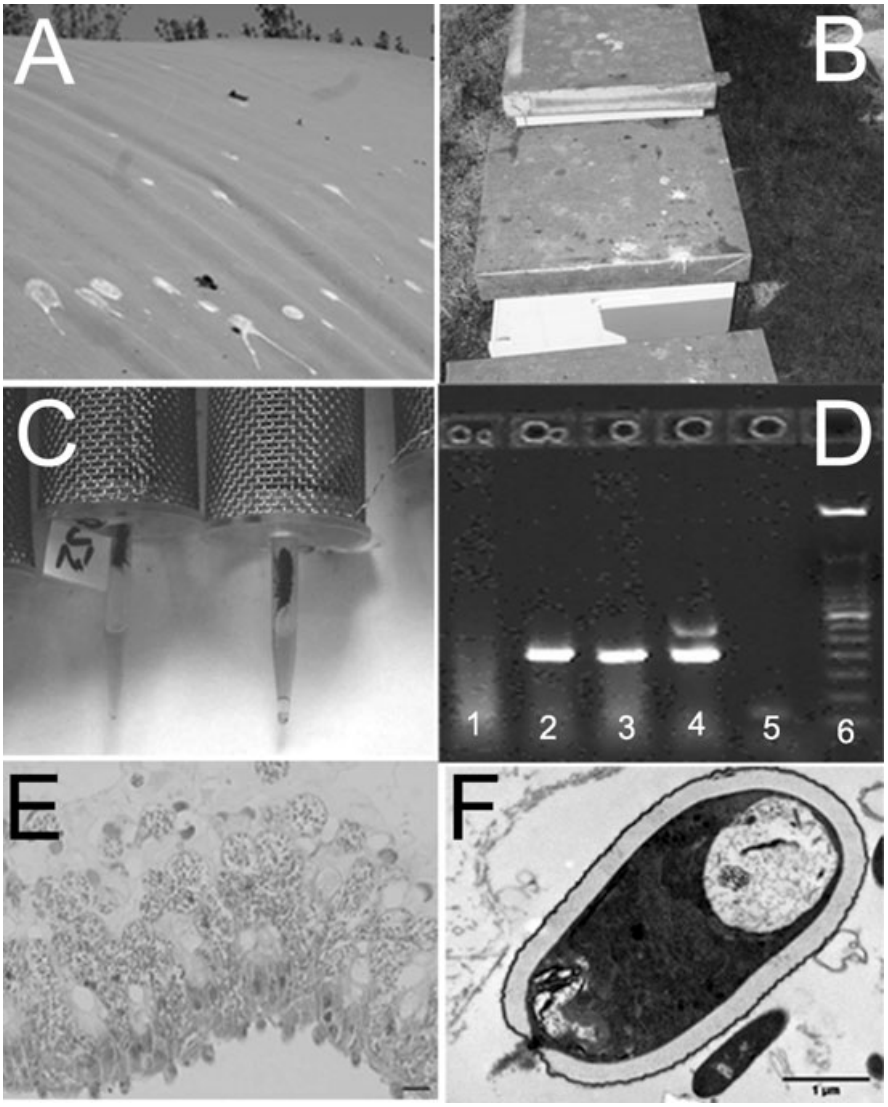


**Fig. 2.** Experiment A: detection and infectivity of spores. Ten regurgitated pellets and four faeces samples of *Merops apiaster* were collected from the cover of *N. ceranae* naturally infected hives (experimental apiaries) and from the greenhouse roof located around apiaries (in Centro Apícola Regional, CAR, Central Spain) on different days on spring months of 2006 and 2007. Samples were aseptically introduced into 50 ml sterile plastic tubes and stored frozen (–20°C) until their analysis. Samples were macerated in $ddH_2O$ and analysed by phase-contrast microscopy (PCM) with 400×. Microsporidia-positive samples were analysed by PCR to determine *Nosema* species using 218MITOC F/R and 321APIS F/R specific primers (Martín-Hernández *et al*., 2007). To confirm spore infectivity, a *Nosema* spore-positive regurgitated pellet collected in May 2006 was macerated and filtered to eliminate remains of the bee exoskeleton. The filtered was centrifuged (800 *g* for 6 min) and re-suspended three times. Sediment was diluted ($ddH_2O$) and the spores were counted to prepare infection doses. Subsequently, sixty 5-day-old *Nosema*-free workers were starved for 5 h in three different cages (20 bees per cage). Two caged bees were each one collectively dosed with 100 000 spores in 1 ml of sucrose (50% w/w in water), and 2% of Promoter L (Calier lab.) for 24 h (Higes *et al*., 2007a,b). Bees of the third cage ($n = 20$) were the uninfected control and were fed with 1 ml of plain sucrose + Promoter L solution. Once the total dose was consumed, all bees were fed *ad libitum* with the same control food. Bees were checked daily and the food was renewed. One bee per cage was sacrificed on days 3, 7 and 21 p.i. and each abdomen was macerated individually to check for infection. Spores were counted (Cantwell, 1970) in PCM 400× magnification and PCR (Martín-Hernández *et al*., 2007) to confirm *Nosema* species. On day 20 p.i., one bee was taken from each of the infected cages and also from the control cage and the ventriculi were fixed with buffered formaldehyde 10% and H&E stained or with 2% glutaraldehide for transmission electron microscopy (Higes *et al*., 2007a).
A. Regurgitated pellets and faeces of *M. apiaster* on greenhouse roof.
B. Faeces of *M. apiaster* on hive's cover.
C. Caged bees collectively fed with *N. ceranae* spores.
D. Polymerase chain reaction for *Nosema* infection and species confirmation (lane 1 = negative reaction in uninfected cage; lanes 2 and 3 = *N. ceranae* bands from infected cage; lane 4 = PCR-negative control; lane 5 = molecular weight marker).
E. Ventricular cells plenty of spores (bar 2 μm).
F. *Nosema ceranae* germinating spore.

 

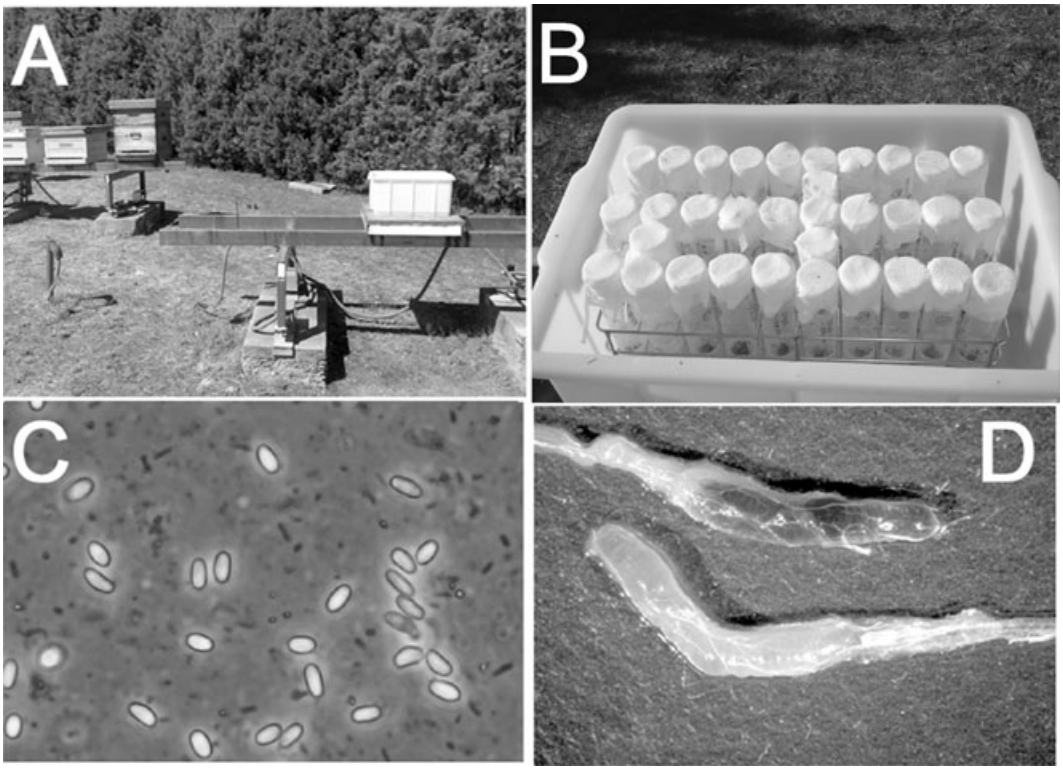


**Fig. 3.** Experiment B: parasitic load and spore viability. Thirty-one regurgitated pellets were collected individually on the same locations as in Experiment A (hive covers and greenhouse roof located in CAR) in order to study the parasitic load of pellets and the viability of spores. All pellet samples were individually weighed and one-third aliquot of each one was examined. The remaining portion of each sample (two-thirds) was placed individually in a 50 ml tube covered by a cotton mesh and they were placed in a neighbouring apiary. All of them were subjected to normal climatic conditions in this location (A and B). Also, two tubes with 1 ml of a water solution containing 2 270 000 *N. ceranae* spores purified in 95% isotonic Percoll® were placed alongside acting as control tubes. A portion of each regurgitated pellet and purified spores was taken after 8 and 18 days and processed to determine parasitic load and viability. Afterwards, the solution from four of the regurgitated pellets (from the last third) was mixed and processed as described above in Experiment A to check its infectivity. The final concentration was established in 3000 spores $\mu l^{-1}$. Thirty-six bees (three cages with 12 bees per cage) were individually infected (Higes *et al.*, 2007a), with 2 μl of solution, and another three control cages (12 bees per cage) were left as uninfected and controls were fed with 2 μl of sucrose solution. Bees were euthanized at 13 days p.i. and their abdomens were processed for PCM (C at 400× magnification) and PCR following the methodology described above. (D) Ventriculi from healthy bee (upper) and from *N. ceranae*-infected bee (lower).

**Table 2.** Pellet samples ($n = 31$) collected on Experiment B were individually weighed.

| | Parasitic load (×100000) per pellet gram | | % Viability | |
|---|---|---|---|---|
| | Regurgitated pellets (mean ± SD) | Control | Regurgitated pellets (mean ± SD) | Control |
| Day 0 | 364.3 ± 377.9 | 22.7 | 89.3 ± 14.9 | 100 |
| Day 8 | 210.2 ± 335.4 | 5.5 | 82.7 ± 28.8 | 100 |
| Day 18 | 75.4 ± 102.3 | ND | 91.3 ± 15.5 | ND |

Parasitic load (as in Cantwell, 1970) and viability (Trypan Blue 0.4% method) were determined at day 0, 8 and 18 after collection on one-third aliquot of each one. Two control tubes (1 ml of a water solution of 2 270 000 *N. ceranae* purified spores) were examined at the same time. ANOVA analysis was made on percentage viability. Parasitic load is expressed as number of spores per pellet gram in 1 ml. ND means no data registered due to the breakage of a tube.

percentage of *A. mellifera.* The variation in diet mainly reflects seasonal, annual and geographical changes of insect fauna as well as temporary or local exploitation of particular species. Despite seasonality, geographic and annual variations, honey bees and bumble bees constituted the main part of the diet in most sites, including, on average, 30–50% honeybees and 21% bumble bees (Fry, 1983; Martínez, 1984; Cramp, 1985). The presence of *Nosema* spores in the regurgitated pellets may have an important role in the transmission of the Microsporidia. Bee-eaters preyed on thousands of forager bees (Galeotti and Inglisa, 2001), which is the bee population that contains the highest spore burdens (R. Martín-Hernández, unpublished). The birds usually expel the pellets around the nest and feeding areas but can also expel them while flying. Adult bee-eaters spend almost half their time flying looking for food, and the spores ingested with the infected bees could be dispersed over long distances. In captive

 

birds, regurgitated pellets are ejected every 1.30–4 h after eating (revised by Cramp, 1985). Thus, the spreading of spores can easily be done during long migration journeys due to diurnal migrant's tendency to often stop their journey to hawk insects before continuing (revised by Cramp, 1985). Apiaries are usually stop-over sites during migration of *M. apiaster* corridors (Yosef *et al*., 2006), most of them critically located and used year after year. Local dispersal of spores can also occur due to their wide feeding area, around 5 km of the gregarious colony nests (revised by Cramp, 1985).

Recent data on the breeding of *M. apiaster* in northern areas as previously recorded (Volet and Burkhardt, 2006) can be related to climatic changes and could have important consequences if birds are considered to be epidemiological dispersal source of pathogens.

While spores dispersed through regurgitated pellets of bee-eaters can have epidemiological consequences, the ethological behaviour of bees in the presence of bee-eaters must also be considered. Foragers spend much more time inside the hive when a bee-eater is outside thus enhancing the risk of infection of interior bees.

The rapid, long-distance dispersal of *N. ceranae* has been attributed to the transport of infected honey bees by commercial or hobbyist bee keepers (Klee *et al*., 2007) but there may be other alternatives, non-exclusive causes, like the one here proposed.

The demonstrated viability of spores inside the regurgitated pellets indicates that they can act as fomites of infective spores. The flying behaviour of bee-eaters can spread them all over long distances. In the same way of the importance of bee-faeces, honey and cadavers as reservoirs of infective *Nosema* spores (OIE, 2004), the significance of other sources like corbicular pollen (Higes *et al*., 2007b) or regurgitated pellets must be considered in the epidemiology of this disease. The fact that bee-eaters are ingesting a large number of infected foragers must be taken into account as a factor that reduces the most affected population and is, someway, even beneficial for the colony.

## Acknowledgements

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# CAPÍTULO II

## Nuevos métodos de control de las nosemosis de *A. mellifera*

La abeja melífera es un animal de renta, por lo que la alteración en su estado sanitario trae consigo una disminución en la vitalidad, productividad y capacidad polinizadora, afectando como consecuencia a la rentabilidad de las explotaciones apícolas y a la biodiversidad. Por ello, el tratamiento y control de las enfermedades infecciosas de las abejas es un aspecto esencial del manejo de las explotaciones apícolas para mantener su papel como productores de alimentos (miel, polen, etc.) y como polinizadores de cultivos y vegetación silvestre.

Las nosemosis causadas por *N. ceranae* y/o *N. apis* son enfermedades de amplia dispersión en las colonias de abejas melíferas de todo el mundo que alcanzan valores de prevalencia muy elevados en algunas regiones, sobre todo en el caso de la nosemosis tipo C. Debido a las repercusiones negativas de las nosemosis tanto para la abeja individual como para las colonias, se hace indispensable la práctica de tratamientos o mecanismos zootécnicos eficaces para el control de estas enfermedades tan extendidas y prevalentes en las colonias de abejas.

De este modo, en el Artículo 9 se estudia la posible eficacia de un mecanismo zootécnico como método alternativo a la aplicación de moléculas activas para el control de las nosemosis. Para ello, se realizó el seguimiento de colonias infectadas de manera natural por *Nosema* spp. (principalmente *N. ceranae*) y se comparó el efecto de la aplicación de fumagilina con el provocado por el reemplazo inducido de la abeja reina en las colonias infectadas como método alternativo de control, teniendo en cuenta que el papel de la reina en la renovación de la población y el reemplazo de las abejas muertas a causa de la infección por *Nosema* es vital para mantener la homeostasis de la colonia.

Por otro lado, en el caso de la aplicación de antibióticos como la fumagilina, distintos estudios previos han revelado la aparición de diferencias en la eficacia de este medicamento en función de la posología utilizada, así como la alta probabilidad de reinfección en las colonias tratadas. Por ello, en el Trabajo Experimental 1 se evalúa la periodicidad adecuada de aplicación de fumagilina para controlar las nosemosis a largo plazo en las colonias infectadas por *Nosema* spp. Esta evaluación se realizó a través del seguimiento de la parasitación en las colonias infectadas y de los parámetros de

vitalidad y productividad presentados por las mismas, identificando los signos clínicos y sub-clínicos mostrados por las colonias con nosemosis (principalmente nosemosis tipo C) a través de la comparación con aquellas no infectadas o con niveles de infección bajos.

Para finalizar, debido a la prohibición en Julio del 2008 por parte de la Agencia Española del Medicamento y Productos Sanitarios (AEMPS) sobre la aplicación del antibiótico fumagilina en las colonias de abejas en nuestro país, se hace necesaria la búsqueda de tratamientos alternativos a este medicamento para el control de las nosemosis. Con el fin de aportar alternativas a este tratamiento, en el Trabajo Experimental 2 se realiza el seguimiento de colonias infectadas de manera natural por *Nosema* spp. (principalmente *N. ceranae*) y tratadas con tres posibles agentes terapéuticos (Nosestat®, Salicilato de Fenilo y Vitafeed Gold®), comparando su acción con la mostrada por la fumagilina, cuya eficacia ha sido anteriormente probada para el control de las nosemosis.

# Artículo 9

## Efecto del cambio de reina inducido sobre la infección por *Nosema* spp. en las colonias de abejas melíferas (*Apis mellifera iberiensis*)

Cristina Botías, Raquel Martín-Hernández, Joyce Días, Pilar García-Palencia, María Matabuena, Ángeles Juarranz, Laura Barrios, Aránzazu Meana, Antonio Nanetti y Mariano Higes

*Environmental Microbiology (2012) 14(4): 845-859*

*Las microsporidiosis de las abejas adultas causadas por* Nosema apis *y* Nosema ceranae *son enfermedades muy comunes en todo el mundo, trayendo consigo efectos negativos sobre la vitalidad y la productividad en las colonias de abejas infectadas. En la actualidad, las opciones disponibles para controlar estas enfermedades son escasas.*

*Por otro lado, el papel de la abeja reina en la renovación de la población en las colonias de abejas y el reemplazo de las abejas muertas a causa de la infección por* Nosema *es vital para mantener la homeostasis de la colonia. Las abejas reinas jóvenes tienen una mayor capacidad de poner huevos y son capaces de producir una mayor proporción de abejas recién emergidas libres de infección para compensar la pérdida de abejas adultas. Por ello, este estudio fue desarrollado para determinar el efecto del cambio de reina inducido sobre la evolución de la infección por* Nosema *spp. en las colonias de abejas. Para ello se hizo un seguimiento de la vitalidad y la producción de miel de las colonias. Además, se evaluó el impacto de la infección a largo plazo en las colonias sobre el estado en los ovarios y ventrículos de las abejas reina.*

*El cambio de reina inducido dio lugar a una reducción notable en las tasas de infección por Nosema, comparables a las provocadas por el tratamiento con fumagilina. Sin embargo, se observaron ciertos efectos perjudiciales en el estado general de las colonias, siendo éstos debidos al efecto combinado de factores de estrés como la falta de reina en la colonia de forma temporal, la falta de cría asociada y una alta tasa de infección por* Nosema *spp.*

*Los ovarios y ventrículos de las reinas procedentes de las colonias infectadas no revelaron signos de infección por* Nosema *spp. y no se apreciaron lesiones en las ovariolas o signos de infección en las células epiteliales del ventrículo.*

***PRESENTACIÓN EN CONGRESO:***

*Botías C, Martín-Hernández R, Meana A, Higes M. Effects of queen supersedure in* Nosema *spp. infected honeybees (*Apis mellifera iberiensis*) colonies. 4º Congreso EurBee, 7-9 de Septiembre 2010, Ankara (Turquía).*

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Environmental Microbiology (2012) **14**(4), 845–859 doi:10.1111/j.1462-2920.2011.02647.x

# The effect of induced queen replacement on *Nosema* spp. infection in honey bee (*Apis mellifera iberiensis*) colonies

**Cristina Botías,[1] Raquel Martín-Hernández,[1,2] Joyce Días,[3] Pilar García-Palencia,[4] María Matabuena,[3] Ángeles Juarranz,[3] Laura Barrios,[4] Aránzazu Meana,[5] Antonio Nanetti[6] and Mariano Higes[1]***

[1]*Laboratorio de Patología Apícola, Centro Apícola Regional, JCCM, 19180 Marchamalo, Spain.*

[2]*Instituto de Recursos Humanos para la Ciencia y la Tecnología (INCRECYT). Fundación Parque Científico y Tecnológico de Albacete, Spain.*

[3]*Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain.*

[4]*Statistics Department, CTI. Consejo Superior de Investigaciones Científicas (CSIC), 28006 Madrid, Spain.*

[5]*Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.*

[6]*Consiglio per la Ricerca e la sperimentazione in Agricoltura, Unità di ricerca di apicoltura e bachicoltura, CRA-API, Via di Saliceto 80, 40128 Bologna, Italy.*

## Summary

**Microsporidiosis of adult honeybees caused by *Nosema apis* and *Nosema ceranae* is a common worldwide disease with negative impacts on colony strength and productivity. Few options are available to control the disease at present. The role of the queen in bee population renewal and the replacement of bee losses due to *Nosema* infection is vital to maintain colony homeostasis. Younger queens have a greater egg laying potential and they produce a greater proportion of uninfected newly eclosed bees to compensate for adult bee losses; hence, a field study was performed to determine the effect of induced queen replacement on *Nosema* infection in honey bee colonies, focusing on colony strength and honey production. In addition, the impact of long-term *Nosema* infection of a colony on the ovaries and ventriculus of the queen was evaluated. Queen replacement resulted in a remarkable decrease in the rates of *Nosema* infection, comparable with that induced by fumagillin treatment. However, detrimental effects on the overall colony state were observed due to the combined effects of stressors such as the queenless condition, lack of brood and high infection rates. The ovaries and ventriculi of queens in infected colonies revealed no signs of *Nosema* infection and there were no lesions in ovarioles or epithelial ventricular cells.**

Received 17 August, 2011; revised 14 October, 2011; accepted 23 October, 2011. *For correspondence. E-mail mhiges@jccm.es; Tel. (+34) 949 250 026; Fax (+34) 949 250 176.



## Introduction

Microsporidia comprise a large phylum of fungal-related pathogens that have been widely studied over the last decade (Keeling and Fast, 2002; Texier *et al.*, 2010). These pathogens are unicellular spore-forming obligate intracellular parasites of all major animal lineages, including humans, insects and fish (Didier and Bessinger, 1999; Kent and Speare, 2005; Williams, 2009; Valles *et al.*, 2011). *Nosema ceranae* was recently identified as a microsporidian parasite of *Apis mellifera* (Higes *et al.*, 2006; Huang *et al.*, 2007) and microsporidiosis due to this pathogen now represents a major disease of honey bees in some climates (Higes *et al.*, 2008; Heintz *et al.*, 2011). The incidence of this disease has increased dramatically in many regions of the world (OIE, 2010), prompting a recent reconsideration of its sanitary status by the World Organization for Animal Health (OIE, 2011). Nevertheless, differences in virulence at the individual bee level have been described following experimental infection (Higes *et al.*, 2007; Paxton *et al.*, 2007; Chen *et al.*, 2009; Forsgren and Fries, 2010), which may reflect the use of different experimental approaches (Forsgren and Fries, 2010). The severity of infection at the colony level can also vary considerably (Cox-Foster *et al.*, 2007; Higes *et al.*, 2008; Bacandritsos *et al.*, 2010; Bromenshenk *et al.*, 2010; Fries, 2010; Gisder *et al.*, 2010; Williams *et al.*, 2010). In field conditions, at the colony level, infection by *N. ceranae* and its ultimate impact on colony health may be influenced by multiple factors. One of these factors may be related to the honey bee colony homeostasis and the role of the queen in bee population renewal and the rate

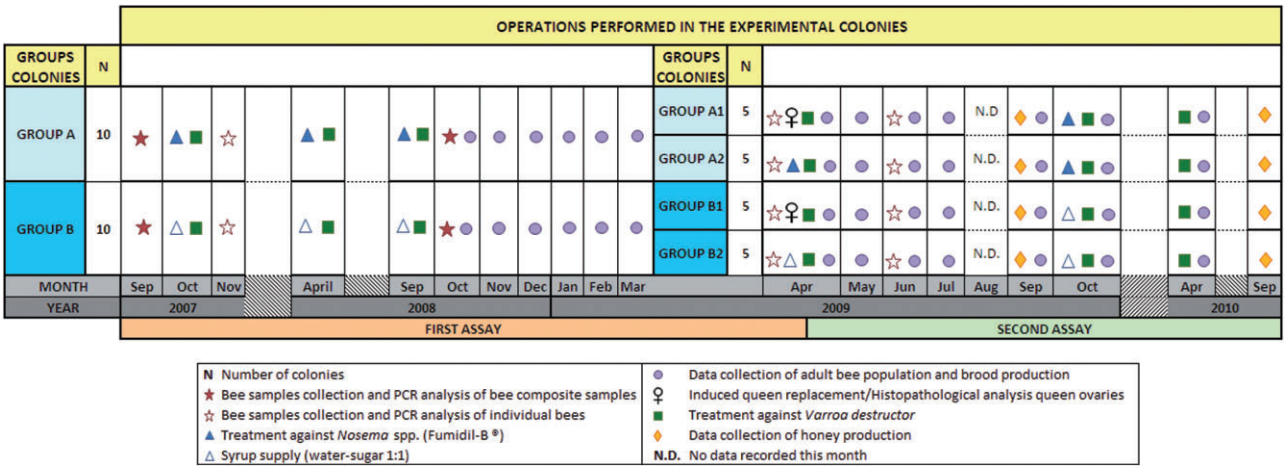


**Fig. 1.** Chronology of the experimental procedures followed in the study.

at which she can replace forager bee losses due to *Nosema* infection (Moeller, 1978; Khoury *et al*., 2011).

Honey bee colonies are composed of related and closely interacting individuals that form a highly complex society (Khoury *et al*., 2011). Colonies feature a haplodiploid sex determination system, whereby queen–worker differentiation is nutritionally determined during the larval stage (Kamakura, 2011). Queens have the longest lifespan, approximately 2 years, although lifespans of up to 8 years have been reported (revised by Page and Peng, 2001). Queens become sexually mature 6 days after emergence and embark upon orientation and mating flights (Remolina and Hughes, 2008). They mate with 10–12 drones (Tarpy and Nielsen, 2002) and store enough sperm to fertilize eggs for their entire lifespan (Woyke, 1960). Sperm remains fertile for several years in the spermatheca of the queen, although in older queens (over 1 year old) spermatozoa move slowly and exhibit a lower metabolic profile (Al-Lawati *et al*., 2009). Vigorous queens lay eggs abundantly (1500–2000 eggs per day) in periods when the pollen supply is high, and they maintain this laying capacity for at least 1 year (Snodgrass, 1956; Winston, 1987; Richard *et al*., 2007; Remolina and Hughes, 2008). Moreover, younger queens have a greater egg laying potential (Woyke, 1984; Prost, 1989; Philippe, 1990; Akyol *et al*., 2007; 2008) and thus, they tend to form larger colonies that produce more honey than those headed by older queens (Woyke, 1984; Harris, 2010) and tend to be less severely affected by certain honeybee diseases such as nosemosis (Moeller, 1978; Pickard and El-Shemy, 1989).

As the queen is the only reproductive female in the colony, her loss means imminent colony death if a replacement is not provided. The replacement of killed or damaged reproductive bees in monogynous colonies is accomplished by the worker honeybees (Butler, 1957; Winston, 1979), who rear replacement reproductive bees from existing immature stages within the first 48 h after queen loss (Fell and Morse, 1984; Hatch *et al*., 1999).

In the present work we describe the effect of induced queen replacement on the development of *Nosema* infection in honey bee colonies in field conditions and on colony strength and honey production. The impact of long-term *Nosema* infection of the colony on the ovaries of the queen was also studied.

## Results

### *First trial: the effect on the queen of colony infection by* Nosema *spp.*

*Evolution of* Nosema *infection in experimental colonies.* In September 2007, all 20 experimental colonies were infected by *N. ceranae*, 10 of which were co-infected with *Nosema apis*.

One month after the first series of treatment (group A, 10 colonies) or vehicle (group B, 10 colonies) applications (see Fig. 1), *Nosema* spp. were no longer detected in 7 of the 10 fumagillin-treated colonies, while the remaining three were still infected with *N. ceranae* alone. The mean percentage of forager bees in group A colonies infected with *N. ceranae* averaged 26.6%. Within the same period, no changes were observed in the infected status of any group B control colonies, which displayed a mean rate of infection of 62.5% (Table 1).

Analysis of the composite forager samples ($n = 20$) in October 2008, after application of fumagillin or vehicle the previous month, revealed that only one fumagillin-treated colony remained infected with *N. ceranae*. By contrast, all group B colonies were still infected by *N. ceranae*, one of which was co-infected with *N. apis* (Table 1).

When the individual forager and house bees were analysed before intervention in April 2009 (Fig. 1), there

**Table 1.** *Nosema* infection during the first trial (queen study) before queen removal in April 2009.

| | | November 2007[a] | | October 2008[a] | April 2008[b] | | |
|---|---|---|---|---|---|---|---|
| Groups colonies | *n* | No. colonies infected | % Forager bees infected | No. colonies infected | No. colonies infected | % Forager bees infected | % House bees infected |
| Group A | 10 | 3 | 26.6 | 1 | 10 | 42 | 4.5 |
| Group B | 10 | 10 | 62.5 | 10 | 10 | 74 | 48.5 |

*n*, number of colonies.
**a.** After application of fumagillin (group A) or syrup (group B).
**b.** Before intervention in the colonies in the spring of 2009).

were more infected bees in the non-treated colonies (group B) than in treated ones (group A), with 74% (Range = 55–95%) of the foragers and 48.5% (Range = 0–80%) of the house bees presenting *Nosema* infection in the group B, whereas 42% (Range = 25–70%) of foragers and 4.5% (Range = 0–15%) of the house bees were infected in group A (Table 1). At this time all the group A colonies were infected with *N. ceranae*, two of which were co-infected with *N. apis*. In group B, all the colonies were infected by *N. ceranae*, one of which was co-infected with *N. apis*.

*Histopathological analysis of queens.* No lesions were observed in the ovarioles or epithelial ventricular cells (Fig. 2C′) of queens removed from colonies from groups A (Fig. 2A–D) or B (Fig. 2A′–D′). In both groups, parallel ovarioles with a normal and well differentiated germarium and vitellarium were observed, and normal differentiation of the ovarioles was evident (Fig. 2A and A′). In general, no cellular disorganization was observed in the follicles, as well as no increase in intracellular spaces and no paracrystalline arrays in the peritoneal epithelium. Healthy follicle cells clustered around the egg cells alternated with nurse cells in a normal pattern. Moreover, the follicle size increased as development proceeded (Fig. 2A,D–A′,D′), and as the eggs approached the oviducts, nurse cells began to shrink and degenerate (Fig. 2B,B′–C,C′). *Nosema* spp. spores were not detected in the ovaries or ventriculi of the queens from either group.

No cell death was evident in the anterior portion of each ovary when TUNEL assays were performed (Fig. 3A,A′), whereas a normal increase in apoptotic cell death was observed in nurse cells (TUNEL positive) that was correlated with the degree of egg maturation (Fig. 3B,B′–C,C′). The number of dead cells did not increase in any of the queen tissues analysed that might be indicative of viral or *Nosema* infection.

### *Second assay: effect of queen replacement on the evolution of* Nosema *infection*

As described in the experimental procedures, each group was further subdivided into two groups, A1, A2, B1 and B2; the letter representing the original treatment group, while the number indicates the queen's status (1 = original queen removed and replaced by newly born queen; 2 = original 2-year-old queen still present). When bee samples collected in April 2009 were analysed before the queen was manipulated (Fig. 1), significant differences were found between the infection rates in foragers from non-treated (groups B1 and B2) and previously treated (groups A1 and A2) colonies. Thus, percentage of infected foragers of groups B1 (73 ± 15.2%, mean ± SD, range = 55–95%; $P < 0.003$) and B2 (75 ± 16.9%, range = 55–95%, $P < 0.05$) were significantly greater than those of groups A1 (46 ± 15.6%, range = 30–50%) and A2 (38 ± 10.9%, range = 25–55%; Fig. 4). The same situation was observed in the case of house bees collected in April 2009, where average of bees infected was significantly higher in groups B1 (42 ± 24.1%, range = 20–70%; $P < 0.05$) and B2 (63 ± 18.9%, range = 0–80%; $P < 0.001$) when compared with those in groups A1 (8 ± 7.6%, range = 0–15%) and A2 (1 ± 2.2%, range = 0–5%; Fig. 5).

Samples were collected from the different colonies in June 2009, after treatment (A2) and syrup (B2) application and after allowing sufficient time for spontaneous requeening and the establishment of a normal brood pattern in the A1 and B1 colonies. When these samples were analysed there were significant differences between groups regarding infection rates of forager bees ($F = 17.2$, $P < 0.0001$). Forager bees from group B2 (control colonies with original queen present) exhibited a significantly higher infection rate (72 ± 12.5%, range 50–80%; $P < 0.0001$) than those from groups A1 (28 ± 2.7%, range 25–30%), A2 (17 ± 12.5%, range 5–35%) and B1 (29 ± 6.5%, range 25–40%). By contrast, no differences in infection rates were observed between groups A1, A2 and B1 (ANOVA, $P > 0.05$). Moreover, contrary to the other groups, the rate of infection in the Group B2 did not significantly decrease in the pre/post-treatment interval (Fig. 4).

House bees exhibited significantly lower rates of infection than forager bees, both in April 2009 ($F = 12.9$, $P = 0.001$) and June 2009 ($F = 14.2$, $P = 0.001$), although intra-group comparisons revealed similar findings to those

 

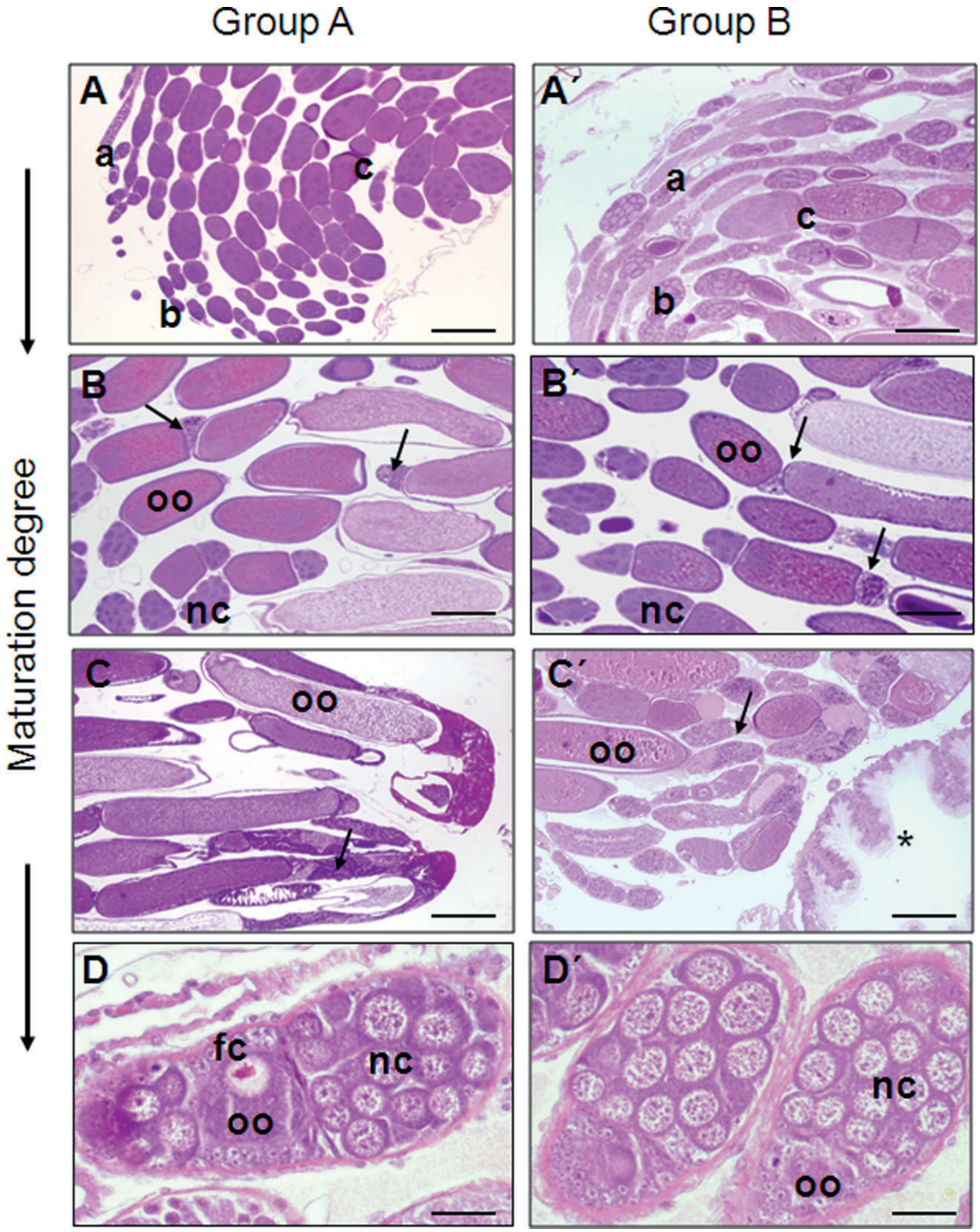


**Fig. 2.** General histology of the ovarioles from group A and B queens showing the germarium (a), the beginning of the vitellarium (b) and a well-differentiated vitellarium (c). Note the disposition of the oocyte (oo) with the follicle (fc) and nurse cells (nc) in the ovariole. Nurse cell death (arrows) is observed as the eggs approach the oviducts. D, D′, Details of the ovarioles showing the oocyte with the follicle and the nurse cells. No *Nosema* spp. spores were detected in ovarioles and ventriculi (asterisk). Scale bars of A,A′–C,C′ = 300 μm, D,D′ = 50 μm.

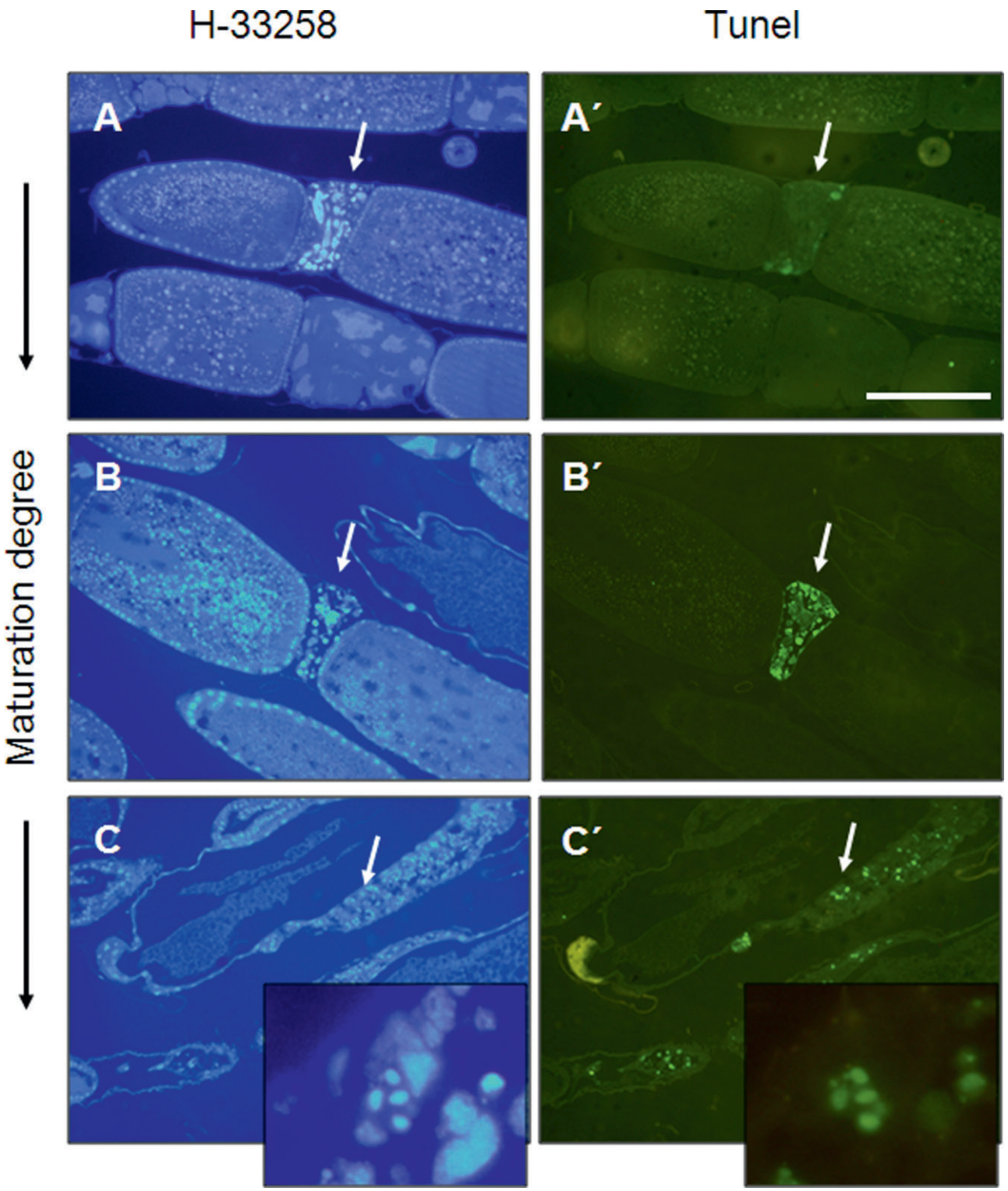


**Fig. 3.** Hoechst staining (H-33258) and apoptotic cells labelled by Tunel reaction in ovaries from queens from groups A (A,A′) and B (B′,B′–C,C′). The amount of clustered Tunel labelled nurse cells increased with egg maturation (A,A′–B,B′) and many dead cells were detected close to the oviducts. The morphology of the apoptotic cells was more evident at higher magnification in C,C′ (inserts). Scale bars = 300 μm.

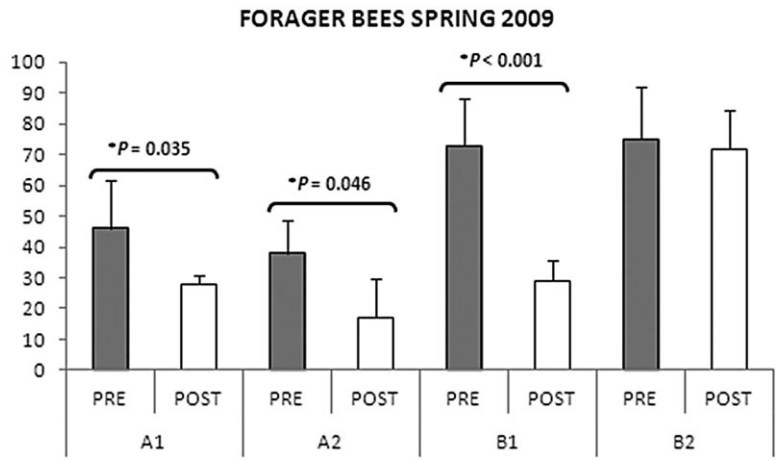


**Fig. 4.** Mean percentage of parasitized forager bees in each group in spring 2009. A1 = queen replacement; A2 = fumagillin application; B1 = queen replacement; B2 = syrup supply; PRE = before fumagillin or syrup application or queen replacement; POST = after fumagillin or syrup application or queen replacement; The asterisk indicates statistically significant differences in the pre–post treatment interval.
[Correction added on 21 December 2011, after first online publication on 28 November 2011: The figure has been replaced to correct the placement of signicant differences.]

of forager bees. It appeared that treatment application (A2) syrup supply (B2) and induced queen replacement (A1, B1) tended to reduce the infection rates in the hive, although this decrease in the pre/post-treatment interval was statistically significant only in the case of group B1 ($P = 0.004$; Fig. 5).

The post-treatment infection rate in group B2 (39 ± 22.2%, range 20–70%) was significantly higher than that of group A1 (0%; $P = 0.003$), A2 (0%; $P = 0.003$) and B1 (4 ± 5.5%, range 0–10%; $P = 0.01$), but it did not differ significantly to that of the pre-treatment situation (63 ± 18.9%, range 40–80%; $P = 0.273$; Fig. 5). Symptoms of chalkbrood disease were observed in two colonies of group B2, one of which collapsed in August 2009 and that left no remaining adults inside the hive. In April 2009, 95% of the forager bees from this colony were infected by *Nosema* spp.

*Adult bee population and brood production.* During the 6 months of the pre-trial period (October 2008–April 2009) the size of the adult bee populations between colonies did not differ significantly (ANOVA, $P > 0.05$; Fig. 6). Between the end of April 2009 and July 2009, a period coinciding with maximum bee activity and honey production, the adult bee populations from groups A1 and A2 were significantly higher than those of groups B1 and B2 (ANOVA, $P < 0.05$). By contrast, no such differences were observed in September and October 2009, although colonies from groups A1 and A2 still presented a trend for higher levels of adult bee population. On the whole there were no significant differences in the brood production between groups (Fig. 7), regardless of the operation applied to them (ANOVA, $P > 0.05$), except for May 2009 when queenless colonies (groups A1 and B1) underwent a broodless period and presented a significantly lower brood production (ANOVA, $P < 0.0001$).

By spring of 2010, 1 year after treatment application and/or queen removal, two colonies had collapsed: one each from groups B1 and B2. Two months before collapse in March 2010, the B1 colony contained only drone brood (colony with laying workers or a drone-laying queen). When both the collapsed colonies were excluded from the comparisons, groups A1 (9.4 ± 1.14 combs with adult bees; $U = 3$, $P = 0.047$), A2 (9.6 ± 0.89 combs with adult bees; $U = 1$, $P = 0.018$) and B1 (9.3 ± 0.58 combs with adult bees; $U = 0$, $P = 0.029$) were significantly more

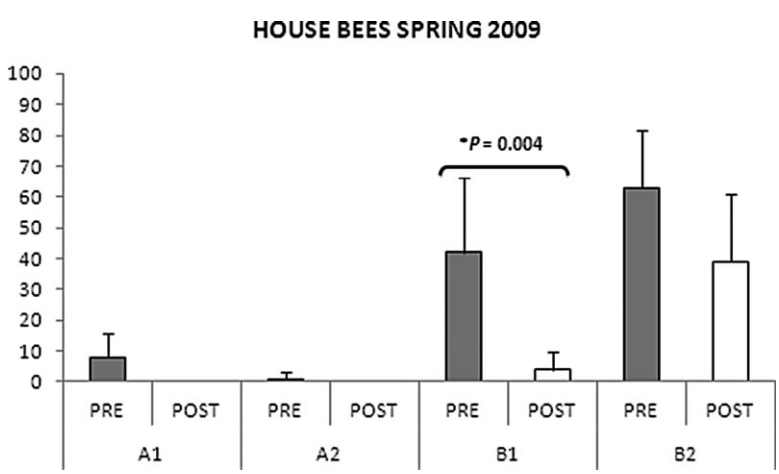


**Fig. 5.** Mean values of the percentage of parasitized house bees in each group in spring of 2009. A1 = queen replacement; A2 = fumagillin supply; B1 = queen replacement; B2 = syrup supply PRE = before fumagillin or syrup application or queen replacement; POST = after fumagillin or syrup application or queen replacement.
[Correction added on 21 December 2011, after first online publication on 28 November 2011: The figure has been replaced to correct the placement of signicant differences.]

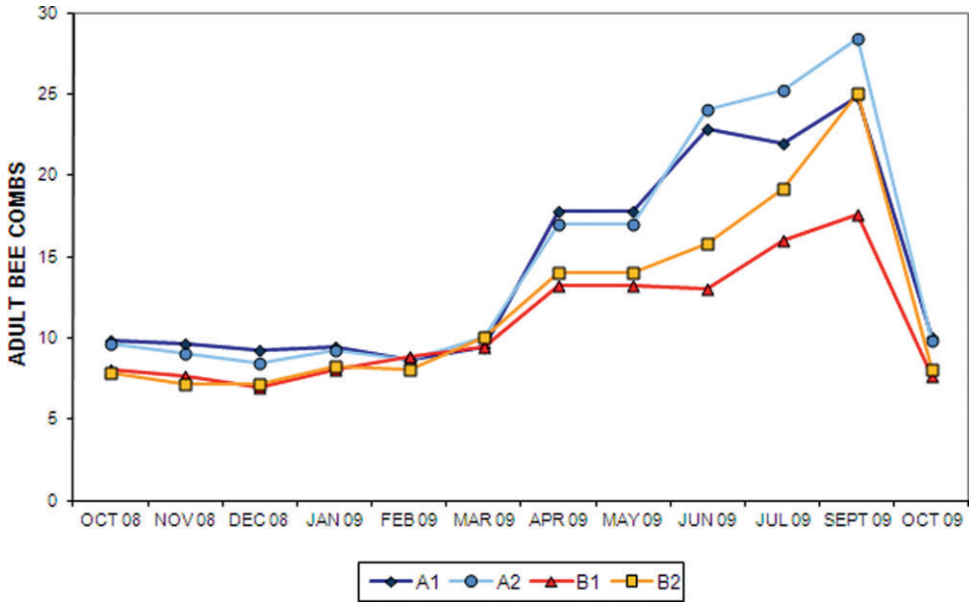


**Fig. 6.** Evolution of adult bee populations in each experimental group of colonies.

populated than the group B2 colonies (7.5 ± 0.58 combs with adult bees; Fig. 8) at this time point. No significant differences in the number of brood cells were found between groups ($\chi^2 = 1.79$, $P = 0.62$).

*Honey production.* There were significant differences in the mean honey production between groups in 2009 ($\chi^2 = 10.3$, $P = 0.016$; Fig. 9), with A2 colonies producing significantly more honey than those of groups B1 ($U = 1$, $P = 0.016$) and B2 ($U = 4$, $P = 0.046$). The highest level of honey was produced by colonies in groups A2 (26.22 ± 7.47 kg per colony) and A1 (20.63 ± 2.79 kg per colony) when compared with that produced by those in groups B2 (18.64 ± 5.23 kg per colony) and B1 (12.10 ± 2.48 kg per colony). One of the colonies of group B1 failed to produce any honey for unknown reasons, while one colony of group B2 produced no honey due to colony death before harvesting. Both non-productive colonies were excluded from the analysis of honey production.

As in 2009, significant differences in honey production were recorded between the experimental groups in 2010

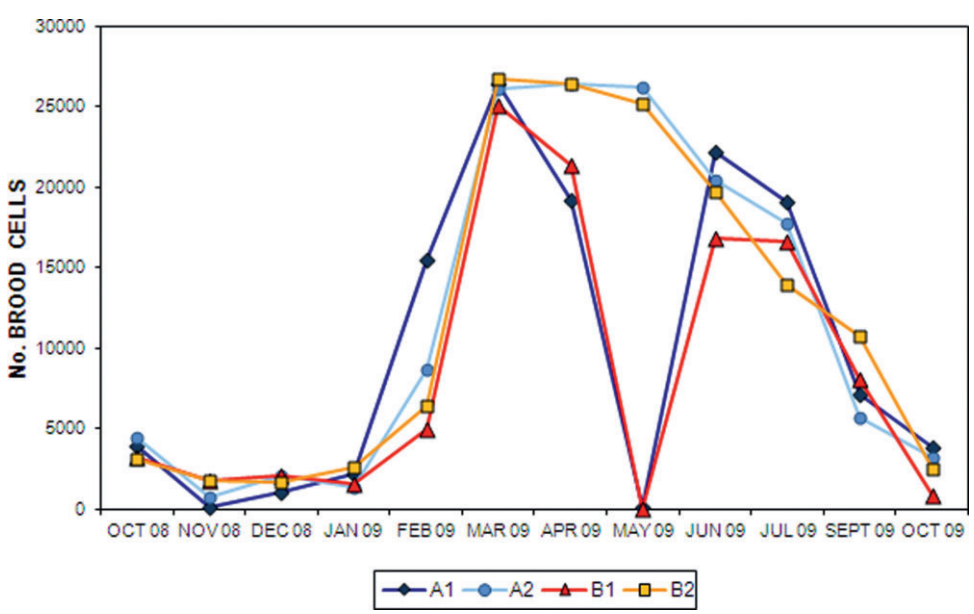


**Fig. 7.** Evolution of brood production in each experimental group of colonies.

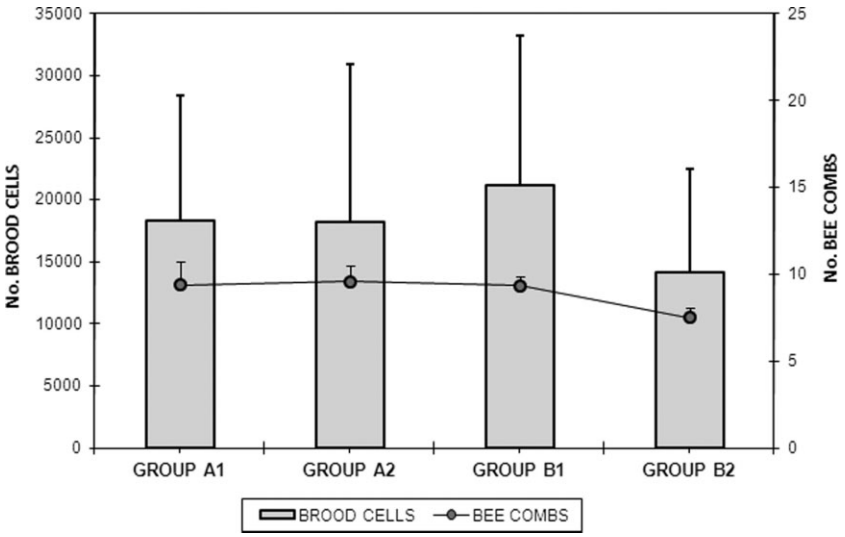


**Fig. 8.** Number of combs covered by adult bees and number of brood cells in each group of colonies in April 2010.

($\chi^2 = 9.3$, $P = 0.026$; Fig. 10). Colonies of group B2 were significantly less productive than those of group A1 ($U = 1$, $P = 0.027$) and A2 ($U = 0$, $P = 0.014$). As in 2009, the highest levels of honey production were recorded in colonies of groups A2 (22.2 $\pm$ 5.71 kg per colony) and A1 (19.29 $\pm$ 4.9 kg per colony). However, unlike the previous year the mean honey production of group B1 (17.98 $\pm$ 9.84 kg per colony) was higher than that of group B2 (9.17 $\pm$ 5.89 kg per colony), although this difference failed to reach statistical significance (Fig. 10).

## Discussion

The present study demonstrates the central role of the queen in the evolution of *N. ceranae* infection of honey bee colonies. Indeed, the removal of the queen and the subsequent replacement with a younger queen decreased the proportion of *Nosema*-infected forager and house bees, which maintained the overall infection at a level compatible with colony viability. This effect should be taken into account when studying the evolution of *Nosema* disease in different geographical areas in which queen replacement (either naturally or by the bee-keeper) frequently occurs. The emergence of brood is the primary natural defence against *Nosema*, allowing replacement of infected bees with healthy young bees, and thus, colonies with vigorous young queens are less prone to suffer *Nosema* infection (Moeller, 1978). Our results are consistent with these observations and the beneficial effects of

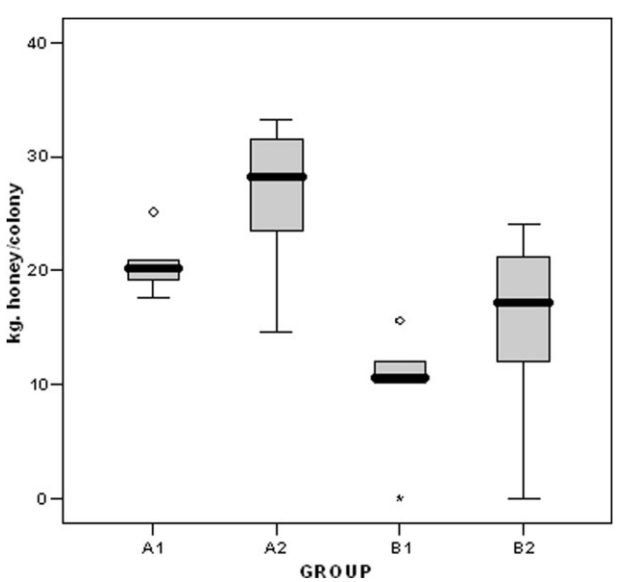


**Fig. 9.** Bloxpot of the amount of honey produced per colony in each experimental group in 2009.

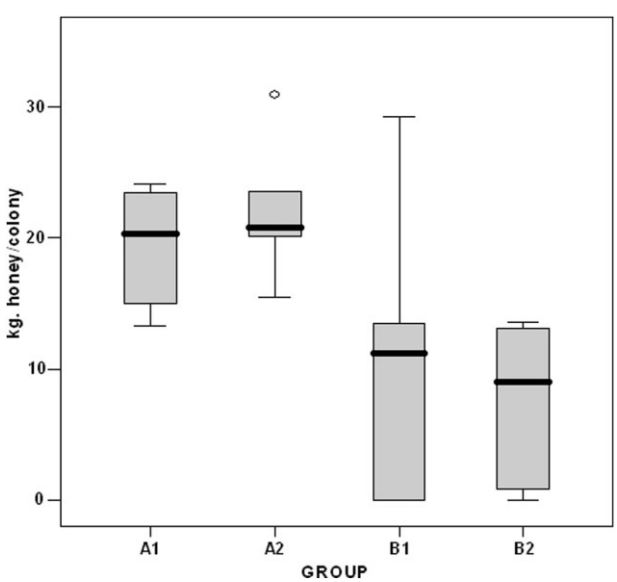


**Fig. 10.** Bloxpot of the amount of honey produced per colony in each experimental group in 2010.

*Nosema* control over the development of bee colonies and the final production of honey are demonstrated in the present assay, because colonies in which nosemosis was controlled were the most vigorous and productive.

The health and fitness of a honey bee colony depends greatly on the quality of the queen (i.e. mating ability, fecundity and offspring viability), as she is the only reproductive female in the colony and she is therefore responsible for the constant renewal of the worker bee population (Gilley *et al*., 2003; Gauthier *et al*., 2011). The age of a queen and the variation in her reproductive potential are important factors influencing the honey bee life cycle (Tarpy *et al*., 2000). Ageing has an impact on the reproductive status of the queen, decreasing her egg-laying potential (Butler, 1957; Philippe, 1990; Akyol *et al*., 2007; 2008) and the quantity and condition of stored sperm (Page and Peng, 2001; Al-Lawati *et al*., 2009). Moreover, honey bee queens undergo physiological changes with age that may affect their pheromone output (Rhodes *et al*., 2007), which in many cases reflects their reproductive status. These subtle differences can be detected by worker honey bees, who are more responsive to queens with a higher reproductive potential, in turn influencing various aspects of their behaviour and physiology (Kocher *et al*., 2009). Hence, to maintain colony health and survival, and to increase strength and productivity, it is essential to ensure the presence of young productive queens in colonies by regular replacement on an annual or biannual basis (Philippe, 1990; Invernizzi *et al*., 2006). This operation may be also beneficial to prevent health problems in the brood and, as demonstrated here, to control infection by *Nosema* and its deleterious effects at the colony level.

*Nosema ceranae* is the aetiological agent of nosemosis type C, one of the most prevalent and economically damaging honey bee diseases under certain conditions (Higes *et al*., 2008; Bacandritsos *et al*., 2010; Bromenshenk *et al*., 2010; Higes *et al*., 2010; Heintz *et al*., 2011). Indeed, the negative effect of infection by *Nosema* (primarily *N. ceranae* in this study) on both final honey production and the bee population are demonstrated in the present assay. At the colony level, parasitization can affect essential colony tasks such as brood care, thermoregulation, defence or foraging (Gauthier *et al*., 2011). In addition, the energetic stress placed upon the host as result of an infection can compromise the immune response itself, allowing other pathogens to invade the host and triggering a cascading effect (Mayack and Naug, 2009). Infection of individual bees by *N. ceranae* has a clear effect at the colony level due to both behavioural changes induced by infection and the continuous death of strongly infected bees (revised by Higes *et al*., 2010). Furthermore, chronically high death rates of forager bees can result in rapid population decline (Khoury *et al*., 2011) and in addition, the plasticity of the age polyethism in colonies means that sustained forager losses result in hive bees joining the foraging population at much younger ages than normal (Huang and Robinson, 1996; Amdam and Omholt, 2003; Woyciechowski and Morón, 2009). However, these precocious foragers are less effective and resilient than normal foragers (Oskay, 2007). These negative effects of *Nosema* infection can be controlled by the application of a *Nosema* treatment or by induced queen replacement, as described in the present study.

In our experimental conditions, the percentage of forager and house worker bees infected by *Nosema* spp. in spring was lower in colonies subjected to induced queen replacement. This effect was similar to that observed following treatment of experimental colonies with fumagillin. Indeed, the high egg-laying potential of younger queens (Moeller, 1978) has previously been shown to successfully control other brood and adult bee diseases (Rahman, 1992; Akyol *et al*., 2007).

The marked effect of queen replacement on the percentage of *Nosema*-infected bees suggests that newly eclosed queens were more efficient in renewing bee populations than older queens, resulting in populations with higher proportions of young bees (Harris, 2008). Moreover, the hiatus in brood production observed in these colonies may have altered the social structure of the colony. This in turn may influence the transmission dynamics of the disease (Naug, 2008), hygienic behaviour (Evans and Spivak, 2010) and/or the vitality and longevity of the bees, with positive effects on colony health. Further studies are required to fully elucidate these potential effects.

In the colonies treated with vehicle solution alone (syrup, group B2), there were no significant changes in either the percentage of infected foragers or house bees. These findings may indicate that supplementary feeding with syrup does not decrease significantly the number of *Nosema*-infected bees, in agreement with earlier reports (Avilez and Araneda, 2007; Hornitzky, 2008).

Interestingly, when composite samples of 30 bees were analysed after the different manipulations in spring 2009, all 20 experimental colonies were infected to some degree, which may have indicated that experimental interventions had no effect on overall colony infection. Nevertheless, significant differences were observed in the percentage of infected bees between groups. Therefore, the analysis of the percentage of infection in each colony rather than in a composite sample proves a more accurate marker of the colonies health status, as reported previously (Doull, 1965; Pickard and El-Shemy, 1989; Higes *et al*., 2008; Meana *et al*., 2010).

There was a lower prevalence of *Nosema* infection in house bees than in foragers from the corresponding colonies, in line with previous reports (L'Arrivée, 1963, Doull, 1965; Meana *et al*., 2010). Colonies treated with fumagil-

 

lin or in which the queen was replaced showed very low (group B1) or no signs of infection in house bees (groups A1 and A2) after operations in spring 2009. This findings suggest that in these colonies with lower *Nosema* infection rates the number of sick bees was inferior, and thus, the overall hive performance and health status may present more optimal levels in comparison with colonies with higher number of infected bees. Moreover, *Nosema* infection has been shown to affect the physiology and behaviour of the infected honey bees, having an impact over their pheromones production (Dussaubat *et al*., 2010), immune response (Antúnez *et al*., 2009), flight behaviour (Kralj and Fuchs, 2010), and inducing an energetic stress (Mayack and Naug, 2009; Martín-Hernández *et al*., 2011a), behavioural fever (Campbell *et al*., 2010) and hunger-mediated conduct (Naug and Gibbs, 2009) at the individual level. Because variation in individual honey bee behaviour and physiology relates to variation in colony state (Schmid-Hempel *et al*., 1993; Khoury *et al*., 2011), all these factors affecting infected bees may cause damage at the social level and have detrimental effects on colony homeostasis, and therefore, the less infected colonies may be less prone to collapse (Higes *et al*., 2008) or to suffer other problems associated with more heavily infected colonies (group B2).

In the present study, induced queen replacement involved the manual removal of the queen from the colony, which was then naturally replaced but resulted in an interim broodless period of approximately 4 weeks. The alterations observed in the size of these colonies (mainly in group B1) were probably due to this brood hiatus. Indeed, this effect was milder in colonies that had been treated the previous autumn with fumagillin (group A1), which probably reflected the strength of the colony, as determined by the adult bee population and *Nosema* infection level. These results suggest that stress, such as the absence of a queen, a lack of brood and high infection rates, may combine to produce detrimental effects on general colony conditions, such as those observed in colonies in group B1.

Colonies that received only syrup in both autumn and spring (group B2) remained highly infected throughout the course of the experiment, and although these colonies were smaller, they produced similar levels of brood to less-parasitized fumagillin-treated colonies (groups A1 and A2). *Nosema ceranae* infection has been previously reported to reduce population size without affecting the brood area (Soroker *et al*., 2011), which may reflect a higher rate of adult bee mortality in strongly infected colonies. A short-term analysis under laboratory conditions reported no differences in survival rates of uninfected versus *N. ceranae*-infected foragers fed *ad libitum* (Mayack and Naug, 2009), and food availability was also suggested to mask the mortality due to the energetic stress imposed by the pathogen (Naug and Gibbs, 2009). These studies may not be comparable with the long-term assays in experimental field conditions described here, as we did find that colonies with higher infection levels that received supplementary sugar syrup had a smaller population and, probably, higher rates of adult bee mortality than colonies with lower infection levels.

As brood production did not significantly differ between the groups, the variations in colony population size, mainly in untreated colonies (groups B1 and B2), are not likely to be due to differences in queen fecundity. Furthermore, histopathological analysis of the queens removed did not reveal any indication of degeneration in the ovaries due to viral infection (Gauthier *et al*., 2011), or signs of *Nosema* infection in the ventriculi and ovaries (Liu, 1992). The decrease in the adult bee population size may be related to the broodless period observed in colonies that lost their queen (group B1) or to the constant loss of infected adult bees due to the pathogenic effects of *Nosema* in the case of the highly infected colonies (group B2; Higes *et al*., 2008; 2009a; 2010; Dussaubat *et al*., 2010; Kralj and Fuchs, 2010; Mayack and Naug, 2010; Suwannapong *et al*., 2010).

Honey production was affected by the sudden pause in brood rearing that followed queen removal and thus, treated colonies of group A2 produced significantly more honey than colonies of group B1 in which the queen was replaced. However, despite queen removal in spring 2009, group A1 colonies produced similar levels of honey to those of A2 in which the queens were untouched.

The negative effects of brood hiatus on the bee population, and on brood and honey production, were no longer evident 1 year after queen removal. Thus, in spring 2010 the colonies that had suffered queen replacement in spring 2009 had adult bee populations and brood and honey production similar to those treated with fumagillin 1 year before. Nevertheless, in the spring 2010, untreated colonies not subjected to queen replacement in 2009 (group B2) exhibited a significantly smaller adult bee population, fewer sealed brood cells and the lowest levels of honey production of all groups. These findings may suggest that colony populations declined when workers died faster than they were replaced (Harris, 2010; revised by Higes *et al*., 2010), in turn affecting honey production. Moreover, greater vitality and honey production was seen in 2010 in the untreated colonies subjected to queen replacement in 2009 (B1) than those in which the queen was untouched (B2), suggesting that colonies headed by younger queens developed faster and attained higher adult bee populations, as previously reported (Harris, 2010).

As nectar and pollen collection depend on the state of the colony, larger colonies can store significantly higher amounts each (Eckert *et al*., 1994). Indeed, we found that

control of *Nosema* infection by fumagillin application in autumn and spring (group A2) resulted in larger and more productive colonies. Similar results were obtained when using a combination of fumagillin in autumn and induced queen replacement in spring (group A1).

The absence of an adequate adult bee population could be associated with some degree of neglect in essential colony tasks such as brood care, thermoregulation, social organization, social 'immunity' and survival (Evans and Spivak, 2010; Gauthier *et al*., 2011). These weaknesses in underpopulated colonies may also alter $CO_2$ levels (Seeley, 1974) and reduce the average temperature of the brood chamber, in turn promoting physiological and behavioural changes in adult bees (Tautz *et al*., 2003; Groh *et al*., 2004; Jones *et al*., 2005) and infection by other opportunistic pathogens (Vojvodic *et al*., 2011). Indeed, such a scenario may explain the chalkbrood infections detected in colonies in which *Nosema* infection was uncontrolled (B2), as reported elsewhere (Hedtke *et al*., 2011). One of these colonies collapsed in August 2009 and was found to contain a high proportion of *N. ceranae*-infected bees and a high proportion of brood affected by chalkbrood disease. It is probable that colony viability was only sustained while the queen could replace the infected adult bees that were dying due to *N. ceranae* infection.

In regions with a rich amount of meliferous flora, such as the Mediterranean area, there is a directly proportional relationship between brood area at the end of winter and honey production some weeks after (Fries, 1988; Prost, 1989). Periodic queen replacement with a young prolific queen helps hives remain strong, healthy and productive, and it is a fundamental element of professional apiculture (Philippe, 1990; Invernizzi *et al*., 2006; Gauthier *et al*., 2011). In our experimental conditions, induced queen replacement in spring significantly decreased the *Nosema* parasitic load on honey bee colonies. While some negative effects of this management practice were observed on bee population and honey production, these had disappeared within 1 year after queen replacement. Queen replacement with a mated queen may have prevented the brood hiatus observed in orphaned colonies, as well as its consequences on the bee population, and on brood and honey production. Further research is required to optimize queen replacement strategies and to build upon the beneficial effects observed in *Nosema*-infected colonies.

In Spain, queen replacement is not a habitual management practice, although in many other countries this is routinely performed every 1 or 2 years. Variations in the severity of *N. ceranae* infection in different countries (Higes *et al*., 2008; Bacandritsos *et al*., 2010; Borneck *et al*., 2010; Bromenshenk *et al*., 2010; Gisder *et al*., 2010) have in general been attributed to differences in the pathogenicity of the microsporidian strains (Williams *et al*., 2010) or to other epidemiological factors (revised by Fries, 2010). We propose that the existing differences in honey bee hive management practices, such as regular queen replacement, may also contribute to this variation in nosemosis type C severity.

## Experimental procedures

### *Honeybee colonies*

Twenty *Apis mellifera iberiensis* colonies located in the experimental apiaries at the 'Centro Apícola de Marchamalo' (CAR) were monitored. All colonies were acquired as nuclei (5 combs with a newly eclosed queen, brood, worker bees and food stores) by the same professional bee-keeper in the spring of 2007. At the start of the experiment, evaluation of brood diseases (American Foolbrood, European Foolbrood and Chalkbrood disease) was performed by confirming the absence of clinical symptoms, and the absence of *V. destructor* and *Nosema* spp. in the colonies was confirmed after analysis according to OIE methods (OIE, 2004) and Martín-Hernández and colleagues (2007) respectively.

The colonies were placed close to another apiary containing colonies naturally parasitized by *N. ceranae* and/or *N. apis*, which acted as a natural source of infection for the new colonies. In September 2007, all the colonies were evaluated for *Nosema* infection, collecting one sample of forager bees ($n \geq 30$) from the entrance of each colony at noon (Meana *et al*., 2010). These 20 composite samples were processed, DNA was extracted and analysed by PCR as described previously (Martín-Hernández *et al*., 2011b). Throughout the study, *V. destructor* was controlled twice annually by treatment with amitraz (Apivar) or flumethrine (Bayvarol) to prevent the negative effects of this parasite on colony health.

### *First assay: the effect on the queen of colony infection by* Nosema *spp.*

*Evolution of* Nosema *infection in experimental colonies.* Upon detection of *Nosema* spp. infection in the 20 colonies (September 2007), each was randomly assigned to two experimental groups of 10 colonies each. The interventions performed in each experimental group are shown in Fig. 1. Briefly, *Nosema* infection was controlled in group A colonies as described previously (Higes *et al*., 2011): 4 applications of 30 mg fumagillin (Fumidil-B) dissolved in syrup (1:1 sugar/water) per hive in October 2007 and April and September 2008. Group B colonies were administered the vehicle alone (syrup, 1:1 sugar/water) in parallel with group A, and thus served as controls. Each group was located in separate apiaries 1 km apart.

The applications were supplied in plastic bags placed over the brood chamber and individual colony consumption was assessed weekly. Any unconsumed syrup was removed before administration of the subsequent dose.

To determine the level of *Nosema* infection after administration of treatment or vehicle, the percentage of infected bees present in every colony was evaluated in November 2007. To that end, a sample of foragers ($n \geq 20$) was col-

lected at the entrance of each of the 20 colonies and the abdomens of the bees were analysed individually ($n = 20$ bees per colony) by putting every single abdomen ($n = 400$) in a well of a 96-well plate (Qiagen) with glass beads (2 mm of diameter, Sigma) and 300 μl of water (PCRq) and subsequently shaking the 96-well plates for 6 min at 30 Hz in a TissueLyser (Qiagen). DNA was extracted and PCR was performed as described previously (Martín-Hernández *et al*., 2011b).

Forager bees were again collected ($n \geq 30$) in the colonies in October 2008 and the 20 composite samples were analysed by PCR as described above.

*Histopathological analysis of queens.* A histopathological study was performed to evaluate *Nosema* infection of the queens and to study the long-term effects of infection in the colony on the queen's ovaries. The queens from five colonies selected at random from each group (Fig. 1) were removed in spring 2009 to allow the colonies to rear an emergency queen from the worker brood present in the combs.

Abdomens of the captured queens were dissected, the ovaries and ventriculi were removed and prefixed for 1 h in a solution containing 2% glutaraldehyde and 2.5% paraformaldehyde, and subsequently they were then washed three times in phosphate buffer (PBS, pH 7.4) and stored at 4°C.

The tissues of interest were embedded in paraffin following standard protocols, and 4 μm sections were stained with haematoxylin-eosin for complete histopathological analysis (as described in Higes *et al*., 2007; 2009b). Apoptosis in the ovaries was determined with the TUNEL assay (Terminal deoxynucleotide transferase mediated X-dUTP nick end-labelling). Briefly, tissue sections (6 μm) that contained all the stages of development of ovarioles were placed on silanized slides, deparaffinized and rehydrated through an ethanol series before rinsing in phosphate buffered saline (PBS). The tissues were treated with Proteinase K (20 mg $ml^{-1}$) for 15 min at room temperature and then with 0.1% (w : v) Triton X-100 in PBS for 10 min. The sections were then incubated in a humidified chamber for 1 h at 37°C in darkness using the *in situ* Cell Death Detection Kit (Roche), according to the manufacturer's instructions. Subsequently, the sections were washed with PBS and mounted in Vectashield (Vector Labs) containing DAPI (1 μg $ml^{-1}$, Sigma) and analysed using an Olympus BX61 epifluorescence microscope equipped with filter sets for fluorescence microscopy (Ultraviolet, UV; 365 nm, exciting filter UG-1). Photographs were obtained with an Olympus DP50 digital camera and processed using Adobe Photoshop 7.0 software (Adobe Systems). Scanning electron microscopy was performed using a Hitachi S-3000N with an energy-dispersive X-ray (EDX) analyzer attached (INCAx-sight, Oxford Instruments).

### *Second assay: effect of queen replacement on the evolution of* Nosema *infection*

This assay began in April 2009, using the same colonies used in the previous trial (see section 2.2) as summarized in Fig. 1.

In spring 2009, the percentage of *Nosema*-infected bees was assessed in samples of both forager ($n \geq 20$) and house ($n \geq 20$) bees from each of the 20 colonies (Higes *et al*., 2008), analysing the bees individually as described previously (Martín-Hernández *et al*., 2011b). Each group was further subdivided into two groups, A1, A2, B1 and B2; the letter representing the original treatment group, while the number indicates the queen's status (1 = original queen removed and replaced by newly born queen; 2 = original 2-year-old queen still present).

The colonies in groups A1 and B1 successfully reared new queens. The colonies of group A2 received four weekly doses per colony of 30 mg fumagillin (Fumidil-B) dissolved in syrup, and B2 colonies received the vehicle alone (syrup) in parallel with colonies of group A2. The first doses of treatment or syrup were administered on the same day as the queen was removed from groups A1 and B1. Syrup consumption was recorded weekly in groups A2 and B2.

To evaluate the effectiveness against *Nosema* of the different treatments and queen replacement, *Nosema* infection of forager and house bees was reassessed after a period deemed sufficient for the newly eclosed queens to restore a normal brood area in groups A1 and B1. Colony strength was estimated on the basis of the number of combs containing adult bees and the number of brood cells (Higes *et al*., 1999). Monthly assessments were carried out from October 2008 to October 2009 (except in August 2009), and a final post-trial assessment was made in April 2010.

Honey production was evaluated in each colony at the end of the harvest season, in September 2009 and September 2010. The frames containing honey from each colony were weighed separately and the total amount of stored honey calculated as the difference in comb weight before and after extraction.

During each colony check, the queen's status and any clinical indications of infections other than *Nosema* were noted.

*Statistical analysis.* The percentage of parasitized bees, the size of the adult honey bee population, the number of brood cells and the amount of honey produced by each experimental group were compared by one-way analysis of variance (ANOVA) or the Kruskal–Wallis test (K–W), depending on the number of observations and the distribution of the data. Where necessary, ANOVA was followed by Bonferroni or Tamhane *post hoc* tests, depending on the homogeneity of variance in each case (Levene's test). The Mann–Whitney *U*-test was used for two-sample comparison in cases where K–W non-parametric tests were used. Differences were considered statistically significant when $\alpha = 0.05$.

Colony strength was reassessed in April 2010, 1 year after treatment of *Nosema* infection and/or queen removal in spring of 2009. The adult bee population and brood area data recorded in spring 2010 were analysed using a K–W non-parametric test ($\alpha = 0.05$) and the Mann–Whitney *U*-test for two-sample comparison. Differences in honey production in each group were determined by the same approach.

All analyses were carried out using SPSS software version 18.0.

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# Trabajo Experimental 1

## Evaluación de la eficacia de distintas posologías para la fumagilina (Fumidil B®) en el tratamiento a largo plazo de las nosemosis en las colonias de abejas melíferas (*Apis mellifera iberiensis*)

*Trabajo experimental no publicado*

*El tratamiento de las enfermedades infecciosas de las abejas es un aspecto esencial en el manejo de las explotaciones apícolas para mantener su papel como productores de alimentos (miel, polen, etc.) y como polinizadores de cultivos y vegetación silvestre.*

*Dada la alta prevalencia y amplia dispersión de* Nosema apis *y* Nosema ceranae *en las colonias de abejas en el ámbito global, se hace indispensable la búsqueda de tratamientos o técnicas apícolas eficaces para el control de estas enfermedades que alcanzan niveles muy altos de prevalencia en algunas regiones del mundo, sobre todo en el caso de la nosemosis tipo C.*

*La fumagilina es el único medicamento registrado en Estados Unidos y en Canadá para tratar las infecciones por* Nosema *spp. en las colonias de abejas melíferas. En la Unión Europea, este antibiótico ha sido utilizado bajo supervisión veterinaria durante varios años por medio de una autorización temporal excepcional, aunque en la actualidad no está permitida su utilización en las colonias de abejas por no estar establecidos los límites máximos de residuos para la sustancia activa.*

*Debido a que estudios previos han revelado la aparición de diferencias en la eficacia de la fumagilina en función de la posología utilizada, así como la facilidad de reinfección en las colonias tratadas, en este estudio se evaluó la periodicidad adecuada de aplicación de fumagilina para controlar la enfermedad a largo plazo en las colonias infectadas por* Nosema *spp. Esta evaluación se realizó a través del seguimiento de la parasitación en las colonias estudiadas y de los parámetros de vitalidad y productividad presentados por las mismas.*

***PRESENTACIÓN EN CONGRESO:***

*Botías C., Martín-Hernández R., Meana A, Higes M. Negative effects of* Nosema *infection in honey production and vitality of honeybee colonies (*Apis mellifera iberiensis*) in Spain. 4º Congreso EurBee, 7-9 de Septiembre 2010, Ankara (Turquía).*

# 1. Material y métodos

## 1.1. Colonias de abejas

En este estudio se hizo el seguimiento de 50 colonias de *Apis mellifera iberiensis* ubicadas en los colmenares experimentales del Centro Apícola de Marchamalo (CAR) desde septiembre del año 2007 hasta diciembre del 2008. A su vez, desde el inicio del ensayo hasta diciembre del 2010 se evaluó cada mes la presencia o ausencia de la reina, la mortalidad de las colonias y los signos clínicos de ciertas patologías de las abejas (nosemosis, varroosis, ascosferosis, loque americana, loque europea). Todas las colonias fueron adquiridas como núcleos (5 cuadros con abejas obreras y una reina) al mismo apicultor en abril del 2007, y se les dio un tiempo para su desarrollo y adaptación a las condiciones de campo de nuestros colmenares experimentales hasta junio del 2007, momento en el que fueron introducidas en nuevas colmenas (10 cuadros con abejas y una reina).

Al inicio del estudio, todas las colonias eran negativas a *Nosema* spp., *Varroa destructor*, loque americana, loque europea y ascosferosis, y presentaban una población homogénea en cuanto a cantidad de población de abejas adultas, área de cría y alimento almacenado. Las enfermedades de la cría (loque americana, loque europea y ascosferosis) fueron evaluadas por medio de la observación de una ausencia de los signos clínicos característicos de estas patologías, mientras que la posible presencia de *V. destructor* se examinó en ácaros en adultos y cría, y la de *Nosema* spp. fue analizada por medio de los métodos descritos en el Artículo 1 de esta memoria (Martín-Hernández y col. ,2012).

Las áreas circundantes a los colmenares no contenían cultivos transgénicos, y tampoco habían sido tratadas con pesticidas como fipronil o imidacloprida.

Las colonias fueron emplazadas cercanas a otro colmenar que contenía colonias parasitadas de manera natural por *N. ceranae* y/o *N. apis*, el cual actuó como fuente natural de infección para las colonias del estudio.

La parasitación por *V. destructor* fue controlada dos veces al año (Apivar®, m.a. amitraz) para evitar los efectos negativos provocados por este parásito sobre el estado sanitario de las colonias en estudio.

## 1.2. Condiciones experimentales del ensayo

Una vez se detectó la presencia de *Nosema* spp. en septiembre del 2007 en las colonias del ensayo (100% positivas a *N. ceranae*; 50% co-infectadas con *N. apis*), las colonias fueron distribuidas al azar en 5 grupos de ensayo, conteniendo cada uno de ellos 10 colonias de abejas. A su vez, estos grupos fueron separados en dos colmenares diferentes con el fin de evitar una posible reinfección de las colonias tratadas a través del contacto con las abejas infectadas procedentes de colonias no tratadas. De este modo, en el Colmenar 1 se instalaron los tres grupos del ensayo que fueron tratados con fumagilina (Fumidil B®; cuatro dosis de 30 mg de fumagilina por colonia administradas 1 vez a la semana: Higes y col., 2011). Tal y como se describe en la Tabla 1, las colonias del grupo 1T recibieron un tratamiento al año (Otoño 2007), las del grupo 2T fueron tratadas dos veces al año (Otoño 2007/Primavera 2008), mientras que las colonias del grupo 4T recibieron 4 tratamientos, uno en cada estación del año (Otoño 2007/Invierno 2008/Primavera 2008/Verano 2008). A su vez, en el Colmenar 2 se instalaron las colonias que no recibieron tratamiento con fumagilina, estableciéndose dos grupos experimentales: en el grupo CS las colonias recibieron jarabe (agua destilada con azúcar en proporción 1:1) en cada una de las estaciones del año (Otoño 2007/Invierno 2008/Primavera 2008/Verano 2008), mientras que las colonias del grupo C (grupo control) no recibieron ningún tipo de sustancia o tratamiento durante el ensayo.

Tanto las dosis de jarabe como las de fumagilina fueron administradas en bolsas de plástico para uso alimentario, depositándose las mismas sobre la cámara de cría en las colonias correspondientes. El consumo de las dosis aplicadas fue evaluado semanalmente (Higes y col., 2011), y la porción de tratamiento no consumida completamente se retiró en el momento de aplicación de la siguiente dosis, procediéndose en estos casos a medir el volumen de producto remanente en la bolsa.

En el caso de las colonias tratadas, la cantidad de fumagilina consumida fue estimada teniendo en cuenta que cada bolsa de tratamiento suministrado en bolsas de 250 ml contenía 30 mg de fumagilina, por lo que en una colonia en la que se hubiese consumido una bolsa completa se habría ingerido esa cantidad de antibiótico.

Desde septiembre del 2007 hasta diciembre del 2010, los signos clínicos indicadores de patologías diferentes a las nosemosis, la presencia de la reina o la mortalidad de las colonias del ensayo se evaluaron en cada una de las visitas a los colmenares.

## 1.3. Seguimiento de la infección por *Nosema* en las colonias

### 1.3.1. Detección de la infección (abejas analizadas colectivamente)

Con el fin de evaluar la infección por *Nosema* spp. en las colonias del estudio, se recogieron muestras de abejas pecoreadoras (n ≥ 30 abejas por muestra) y de abejas de interior (n ≥ 30 abejas) siguiendo los métodos descritos en el Artículo 9 de esta memoria (Botías y col., 2012a). Las abejas pecoreadoras se recogieron en 10 momentos del ensayo, aproximadamente una vez al mes, entre los meses de septiembre del 2007 y diciembre del 2008 (Tabla A1). El ADN de las muestras colectivas (n ≥ 30 abejas) fue extraído y el análisis de PCR fue realizado tal y como se describe en el Artículo 1 (Martín-Hernández y col., 2012).

| INTERVENCIONES EN LAS COLONIAS | Sep 07 | Oct 07 | Nov 07 | Ene 08 | Feb 08 | Abr 08 | May 08 | Jun 08 | Jul 08 | Ago 08 | Sep 08 | Oct 08 | Nov 08 | Dic 08 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aplicación fumagilina | | 1T 2T 4T | | 4T | | | 2T 4T | | | 4T | | | | |
| Aplicación jarabe | | CS | | CS | | | CS | | | CS | | | | |
| Recogida AP y PCR de abejas de manera colectiva | T | T | T | T | T | | T | | T | T | T | | | T |
| Recogida AP y PCR de abejas de manera individual | | T | T | | | | T | | T | | | | | |
| Recogida AI y PCR de abejas de manera colectiva | | T | T | | | | T | | T | | | | | |
| Recogida AI y PCR de abejas de manera individual | | T | T | | | | T | | T | | | | | |
| Recogida de datos población y cría | | T | T | T | T | T | T | T | T | T | | T | T | T |
| Recogida de datos producción de miel | | | | | | | | | | | T | | | |

Tabla A1. Cronología de las intervenciones realizadas en las colonias (AP = Abejas pecoreadoras; AI = Abejas de interior; T = Todos los grupos).

### 1.3.2. Porcentaje de abejas infectadas antes y después de las intervenciones

El porcentaje de abejas obreras infectadas por *Nosema* spp. también se evaluó en cada colonia antes y después de las intervenciones en otoño 2007 y en primavera 2008 con el fin de determinar el efecto de las distintas intervenciones en la evolución de la infección por *Nosema* spp. al principio del ensayo (otoño) y durante el periodo de mayor actividad de las colonias (primavera). Para ello se recogieron muestras de pecoreadoras (n ≥ 20 abejas) y de abejas de interior (n ≥ 20 abejas) de cada colonia siguiendo la metodología descrita previamente (Artículo 9: Botías y col., 2012a), una semana antes de suministrar la primera dosis de tratamiento, y dos semanas tras la aplicación de la última dosis de tratamiento (Tabla A1).

En el caso de las abejas pecoreadoras, el porcentaje de abejas infectadas se determinó a través del análisis individual de 20 abejas procedentes de cada una de las colonias del estudio, siguiendo la metodología descrita en el Artículo 1 (Martín-Hernández y col., 2012).

Para las abejas de interior, el porcentaje de abejas infectadas se halló únicamente en algunas colonias elegidas al azar de entre aquellas que continuaron mostrando infección tras las intervenciones, utilizando los mismos métodos que los descritos para las abejas pecoreadoras.

Además, con el fin de conocer la incidencia de *N. apis* y *N. ceranae* dentro de las colonias infectadas, se comparó la prevalencia de estos dos microsporidios en las abejas pecoreadoras analizadas de manera individual tras las intervenciones de primavera y de otoño.

## 1.4. Seguimiento de los parámetros de vitalidad en las colonias

La población de abejas adultas y la producción de cría se evaluó a lo largo del ensayo (desde octubre del 2007 hasta diciembre del 2008, Tabla A1) analizando los cuadros cubiertos por abejas y cuantificando el número de celdillas de cría operculada (Higes y col., 1999, 2008). Tanto la población de abejas adultas como el número de celdillas de cría fue analizado cada mes (excepto en los meses de diciembre 2007 y marzo y septiembre del 2008), realizando un total de 12 mediciones.

Figura A1. Medición del área ocupada por las celdillas de cría en una de las colonias del ensayo.

La proporción de celdillas de cría (NC) con respecto al número de cuadros cubiertos por abejas (P) también fue calculado mediante la fórmula NC/P.

La producción de miel de cada una de las colonias fue calculada al final de la temporada apícola (septiembre 2008) pesando cada uno de los cuadros con miel de las colonias antes y después de ser extraída y estimando la cantidad producida en cada colonia a partir de la diferencia entre estos dos pesos (Artículo 9: Botías y col., 2012a). Al calcular la producción media por colonia en cada grupo únicamente se tuvieron en cuenta las colonias que permanecían vivas en la etapa de recolección de miel.

Figura A2. Pesaje de la miel contenida en uno de los panales de las colonias del estudio.

## 1.5. Análisis estadístico

Las variables dependientes estudiadas fueron: el porcentaje de abejas infectadas, la cantidad de cuadros con abejas adultas, la cantidad de celdillas de cría, la proporción de celdillas de cría por cantidad de abejas adultas y la producción de miel. Estas variables fueron normalizadas mediantes transformaciones lineales y se tuvieron en cuenta dos factores, un factor fijo (tratamiento) y un factor de medida repetido (efecto del tiempo). Debido a que el modelo reveló que los efectos del tratamiento variaron con el efecto del tiempo, los valores medios de los distintos parámetros (porcentaje de abejas adultas, cantidad de celdillas de cría, proporción de cría por abejas adultas, y la producción de miel) fueron comparados por grupo y punto en el tiempo utilizando un análisis de varianza unidireccional (ANOVA). Cuando fue necesario, el análisis de ANOVA fue seguido por un test post hoc de Bonferroni o de Tamhane, dependiendo de la homogeneidad de la varianza en cada caso (determinado utilizando el test de Levene).

Además, se utilizó la prueba *t* de Student para comparar la prevalencia de *N. apis* y de *N. ceranae* en las abejas pecoreadoras analizadas individualmente tras las intervenciones de otoño y primavera.

En todos los análisis estadísticos, se consideraron como diferencias significativas aquellas en las que $\alpha \leq 0.05$.

Para determinar el grado de dependencia entre el nivel de infección y: (a) la cantidad de población; (b) la cantidad de celdillas de cría; (c) la cantidad de miel producida en las correspondientes colonias; y (d) la cantidad de fumagilina consumida en el caso de las colonias tratadas (grupos 1T, 2T y 4T en otoño 2007, y grupos 2T y 4T en primavera del 2008) se calculó el coeficiente de correlación de Spearman. Estas correlaciones fueron realizadas en función del porcentaje de abejas infectadas en noviembre del 2007 (tras las intervenciones de otoño) y en julio del 2008 (tras las intervenciones de primavera). Las correlaciones fueron consideradas significativas cuando $\alpha \leq 0.05$ (test de dos colas).

Los análisis fueron realizados utilizando el programa informático SPSS 18.0.

# 2. Resultados

## 2.1. Seguimiento de la infección por *Nosema* en las colonias

### 2.1.1. Detección de la infección (abejas analizadas colectivamente)

#### a) Abejas pecoreadoras

Las 50 muestras de abejas pecoreadoras analizadas mostraron la presencia de *N. ceranae* al principio del ensayo en septiembre del 2007, con 25 colonias (50%) presentando además co-infección con *N. apis*. Los análisis de las muestras de abejas pecoreadoras que fueron recogidas a lo largo de todo el ensayo (desde septiembre del 2007 hasta diciembre del 2008) y analizadas colectivamente revelaron la presencia mayoritaria de *N. ceranae* en las colonias positivas a *Nosema* (314 de las 413 muestras positivas a *Nosema*; 76%), aunque también se detectó la co-infección con *N. apis* en algunas de las muestras (97 de las 413 muestras positivas a *Nosema*; 23,5%). Por otro lado, la infección con *N. apis* únicamente fue detectada en 2 de las colonias (0,5%). Ambos microsporidios fueron detectados a lo largo de todo el ensayo, y mientras la presencia de *N. ceranae* fue constante a lo largo del año, la infección por *N. apis* fue más prevalente en los meses de otoño y primavera (Figura A3).

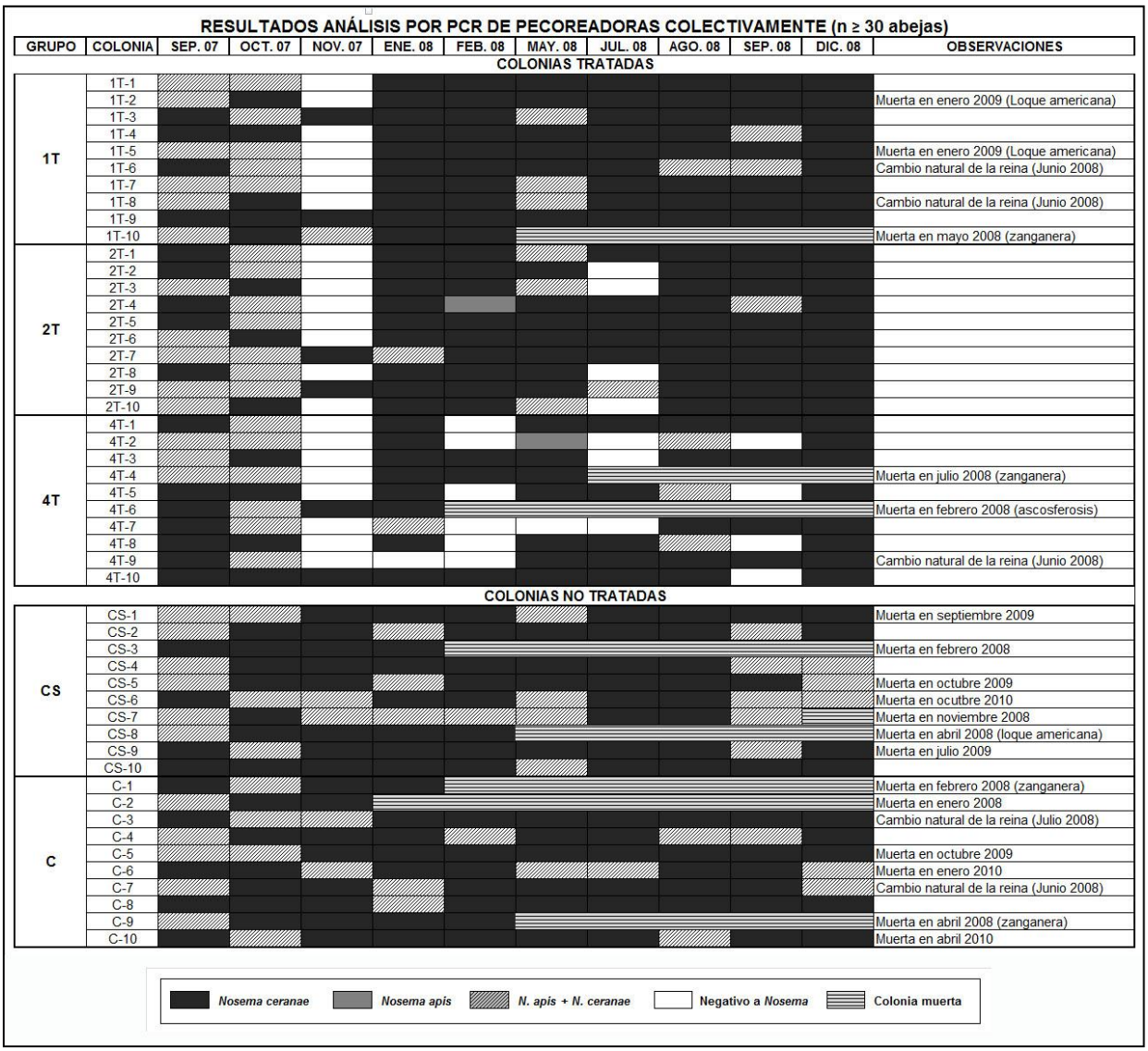



Figura A3**.** Resultados del análisis de PCR de las abejas pecoreadoras analizadas de manera colectiva (n ≥ 30 abejas por muestra) a lo largo del ensayo, y datos sobre otras patologías detectadas, los cambios naturales de la abeja reina y el colapso de colonias.

Todas las colonias no tratadas con fumagilina permanecieron infectadas a lo largo de todo el ensayo, mientras que algunas de las colonias tratadas se mostraron libres de infección tras la aplicación de los tratamientos (Figura A4).

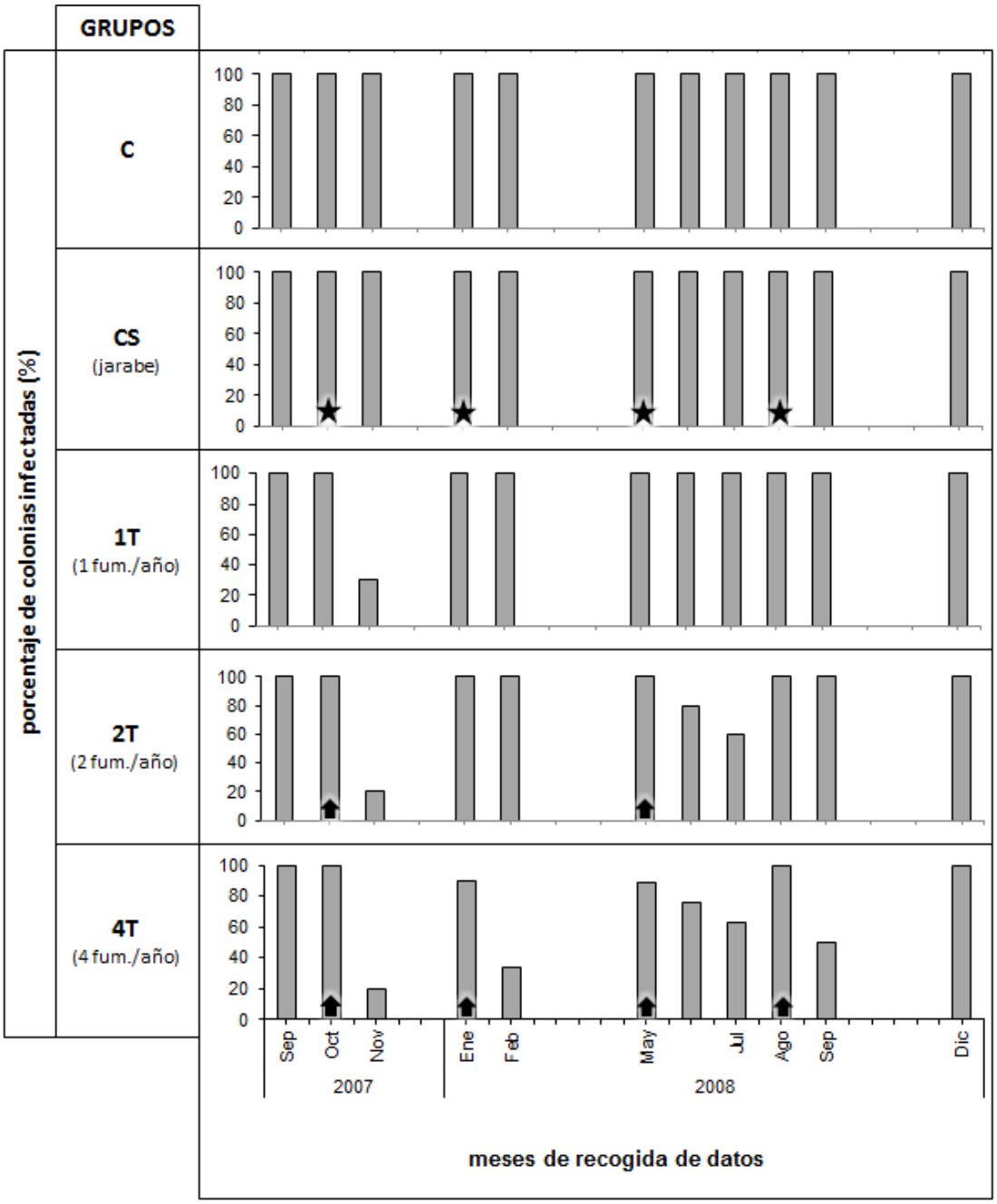
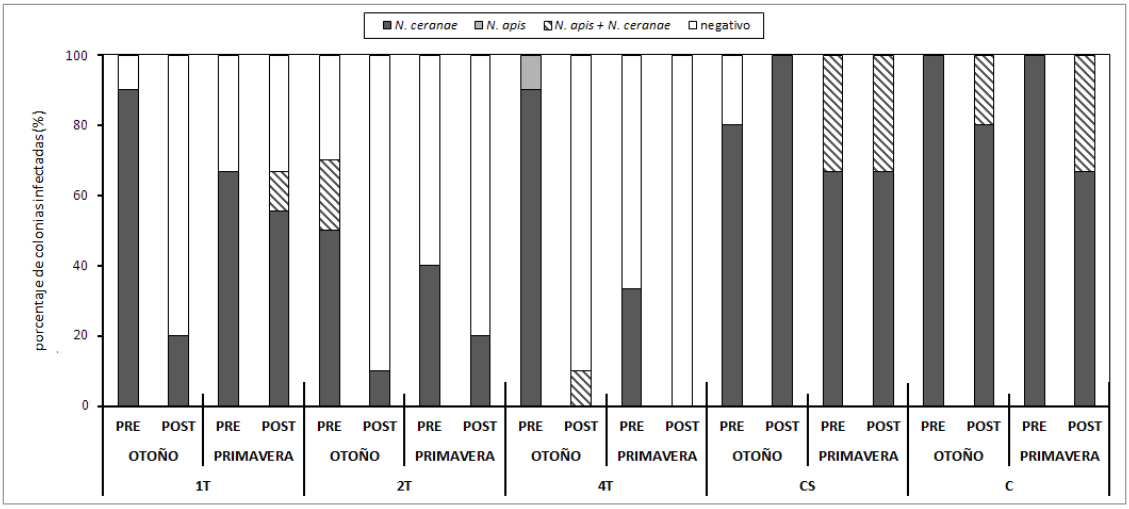



Figura A4. Porcentaje de colonias infectadas por *Nosema* spp. a lo largo del ensayo, en función del análisis de muestras de abejas pecoreadoras analizadas de manera colectiva (n ≥ 30 abejas por muestra). Los puntos en los que se administró fumagilina (flechas) y jarabe (estrellas) son indicados en la figura.

**b) Abejas de interior**

Antes de las intervenciones realizadas en octubre del 2007, se encontró la presencia de *N. ceranae* en 43 de las 50 colonias del estudio (86%; Figura A5), apareciendo este microsporidio en coinfección con *N. apis* en 2 colonias (4%; dos colonias del grupo 2T). Las 5 colonias restantes no mostraron la presencia de *Nosema* spp. (10%; 1 colonia del grupo 1T, 3 colonias del grupo 2T y 1 colonia del grupo CS) en ese punto del ensayo. En función de los resultados obtenidos tras el análisis de las abejas colectivamente (n ≥ 30 abejas) antes y después de las intervenciones de otoño 2007 y primavera del 2008 (Figura A5a), se evaluó el porcentaje de abejas infectadas en algunas de las colonias que permanecieron infectadas por *Nosema* spp. tras las intervenciones (Figura A5b).

**a**




**b**

| GRUPO | COLONIA | PERIODO | Resultado (PCR) análisis colectivo de abejas (n ≥ 30) | Resultado (PCR) análisis individual de abejas (n = 20) | | |
|---|---|---|---|---|---|---|
| | | | | *N. ceranae* | *N. apis* | *N. ceranae* + *N. apis* |
| 1T | 1T-3 | OTO-POST | *N. ceranae* | - | - | - |
| | 1T-6 | PRIM-POST | *N. ceranae* | - | - | - |
| | 1T-7 | PRIM-POST | *N. ceranae* + *N. apis* | - | - | - |
| 2T | 2T-7 | OTO-POST | *N. ceranae* | - | - | - |
| | 2T-6 | PRIM-POST | *N. ceranae* | 1/20 | - | - |
| | 2T-9 | PRIM-POST | *N. ceranae* | - | - | - |
| 4T | 4T-6 | PRIM-POST | *N. ceranae* + *N. apis* | 6/20 | - | 1/20 |
| CS | CS-2 | OTO-POST | *N. ceranae* | 2/20 | - | - |
| | | PRIM-POST | *N. ceranae* | 2/20 | - | - |
| | CS-7 | OTO-POST | *N. ceranae* | 2/20 | - | - |
| | | PRIM-POST | *N. ceranae* | 4/20 | - | - |
| C | C-3 | OTO-POST | *N. ceranae* | 2/20 | 1/20 | - |
| | | PRIM-POST | *N. ceranae* | 6/20 | - | - |
| | C-6 | OTO-POST | *N. ceranae* | 2/20 | - | - |
| | | PRIM-POST | *N. ceranae* | 3/20 | - | - |

Figura A5. Infección por *Nosema* spp. en las abejas de interior

a: Porcentaje de colonias infectadas por *Nosema* spp. en cada grupo antes y después de las intervenciones de otoño 2007 y primavera 2008, basado en el análisis de PCR de las muestras de abejas de interior de manera colectiva (n ≥ 30 abejas por muestra).

b: Resultado de PCR de las muestras de abejas de interior analizadas individualmente (n = 20 abejas de interior) realizado en colonias seleccionadas al azar. Además se muestran los resultados obtenidos al analizar las abejas de manera colectiva (n ≥ 30 abejas) en esas colonias. Los guiones indican que *Nosema* spp. no se detectó en el análisis de PCR de las abejas individuales.

## 2.1.2. Porcentaje de abejas infectadas antes y después de las intervenciones

### a) Abejas pecoreadoras

Antes de la intervención de octubre 2007, al inicio del ensayo, el grupo CS fue el que presentó un menor número de abejas pecoreadoras infectadas (significativamente menor al comparar con los grupos 1T y 4T, F = 4.39, *P* = 0.006; Figura A6). Sin embargo, tras la aplicación de los tratamientos (noviembre 2007), los grupos 1T, 2T y 4T mostraron una menor proporción de abejas infectadas (F = 21.1, *P* ≤ 0.001; Figura A6) en comparación con los grupos CS y C.

La fumagilina provocó una disminución significativa en la proporción de pecoreadoras infectadas de octubre a noviembre en los grupos que la recibieron (1T, 2T y 4T; *P* < 0.001). En cambio, en este mismo periodo, las tasas de infección tendieron a incrementarse en las colonias no tratadas (grupos CS y C: Figura A6), aunque este efecto no resultó ser estadísticamente significativo (*P* > 0.05).

Tras la aplicación de los tratamientos de primavera en las colonias (Tabla 1), los grupos tratados 2T y 4T presentaron una tasa de infección significativamente menor que la registrada en los grupos CS y C (F = 46.3, *P* < 0.001). En el grupo 1T, que había recibido fumagilina únicamente en otoño del 2007, se encontró un porcentaje de abejas infectadas significativamente menor que el encontrado en los grupos no tratados CS (*P* = 0.001) y C (*P* = 0.01), aunque significativamente mayor que el mostrado por los grupos 2T (*P* < 0.001) y 4T (*P* < 0.001: Figura A6).

Dentro de cada grupo, los porcentajes de parasitación antes y después de la intervención de primavera 2008 reflejó una tendencia a bajar en los grupos 2T y 4T, mientras que estos porcentajes subieron en el grupo 1T, CS y C (Figura A6), aunque estas diferencias no fueron estadísticamente significativas (*P* > 0.05). Se encontró una correlación inversamente proporcional entre el porcentaje de abejas infectadas por *Nosema* spp. y el consumo medio de fumagilina en cada grupo (tratamientos de otoño, *r* = -0.54, *P* = 0.021; tratamientos de primavera, *r* = -0.69, *P* < 0.001; coeficiente de correlación de Spearman).

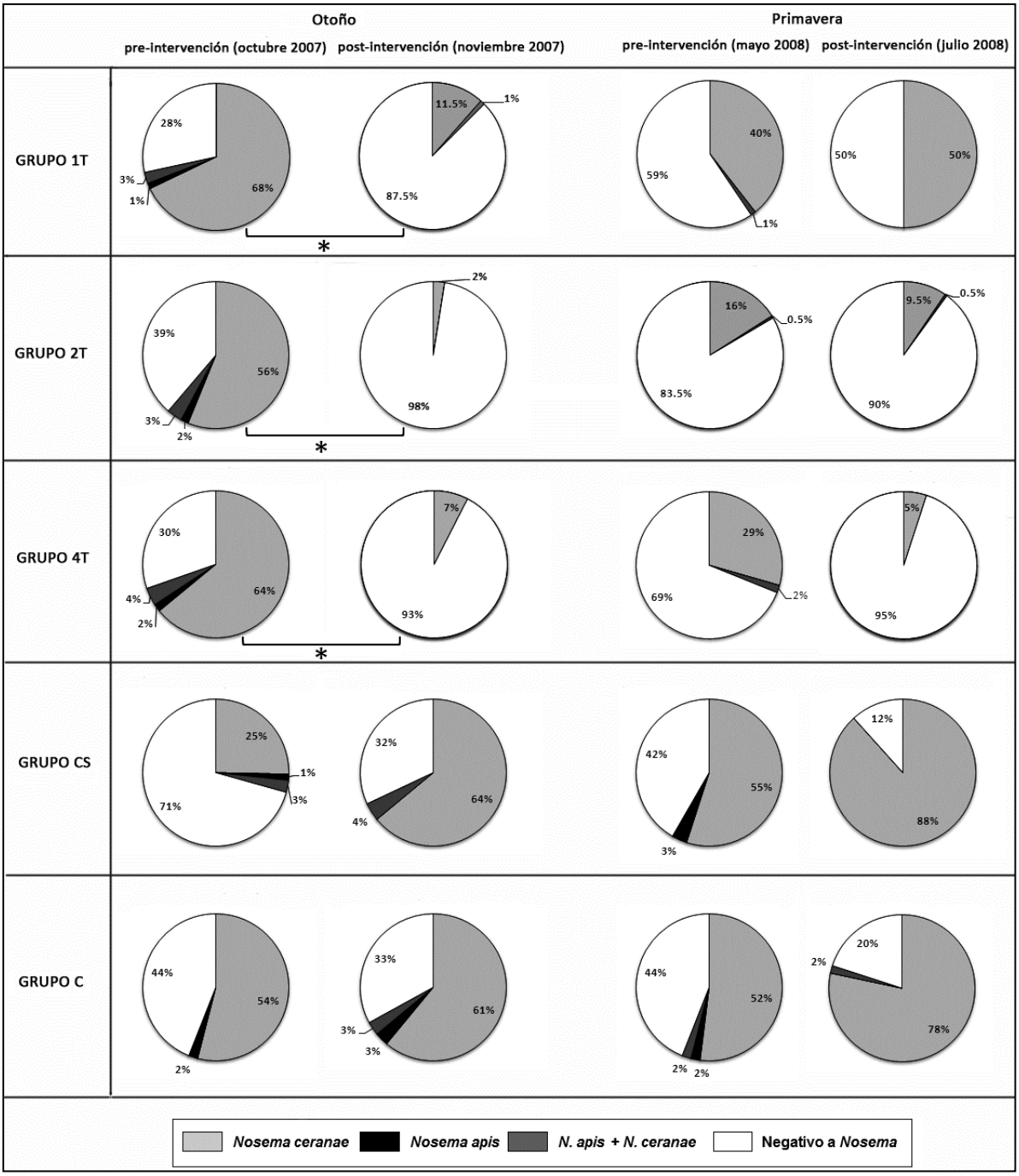



Figura A6. Porcentaje de pecoreadoras infectadas por *N. ceranae* y *N. apis* en cada grupo (n = 200 abejas analizadas individualmente por grupo y punto de muestreo; *i.e.* 20 abejas analizadas de cada una de las 10 colonias pertenecientes a cada grupo). Los análisis se realizaron en otoño 2007 (antes y después de las intervenciones) y en primavera 2008 (antes y después de las intervenciones). Los asteriscos indican la existencia de diferencias significativas ($P < 0.001$) en el intervalo pre-post intervenciones.

Asimismo, la prevalencia de *N. ceranae* fue significativamente mayor que la presentada por *N. apis* en las abejas analizadas de manera individual (Figura A6), tanto en otoño (F = 54.9, $P < 0.001$; prueba *t* de Student) como en primavera (F = 19.3, $P < 0.001$; prueba *t* de Student).

**b) Abejas de interior**

En las colonias tratadas, un porcentaje inferior al 5% de las abejas estaban infectadas por *Nosema* spp. tras las intervenciones de octubre del 2007 y mayo del 2008, excepto en el caso de una colonia (4T-6, con 35% de las abejas infectadas por *N. ceranae* tras el tratamiento de otoño), mientras que. En las colonias no tratadas, los niveles de infección se movieron en el rango del 10-30% (Figura A5b).

## 2.2. Seguimiento de los parámetros de vitalidad en las colonias

### 2.2.1. Población de abejas adultas y cantidad de celdillas de cría

A lo largo del estudio, la población de abejas adultas fue mayor en aquellas colonias que recibieron tratamiento con fumagilina.

Al principio del ensayo (octubre 2007) la población de las colonias de los distintos grupos presentó un tamaño similar (F = 0.3, $P = 0.8$), con una bajada más pronunciada y sostenida en el tamaño poblacional de los grupos CS y C desde el inicio hasta febrero del 2008. En ese momento del ensayo, las colonias de los grupos tratados (1T, 2T, 4T) mostraron una población significativamente mayor (F = 17.8, $P < 0.001$) que la de los grupos control (CS y C: Figura A7).

También durante los meses de primavera y verano (de abril a agosto), las colonias de los grupos 2T y 4T fueron significativamente más pobladas que las de los grupos control (CS y C, $P < 0.001$). A su vez, las colonias del grupo 1T (únicamente tratadas en otoño del 2007) presentaron una población significativamente mayor que las del grupo C en julio ($P = 0.04$) y en agosto ($P = 0.02$).

La población máxima fue alcanzada en el mismo mes en todos los grupos del ensayo (julio 2008: Figura A7).

Además, en julio del 2008 se encontró una correlación negativa entre el tamaño poblacional y el porcentaje de abejas parasitadas (r = - 0.57, *P* = 0.001; coeficiente de correlación de Spearman).

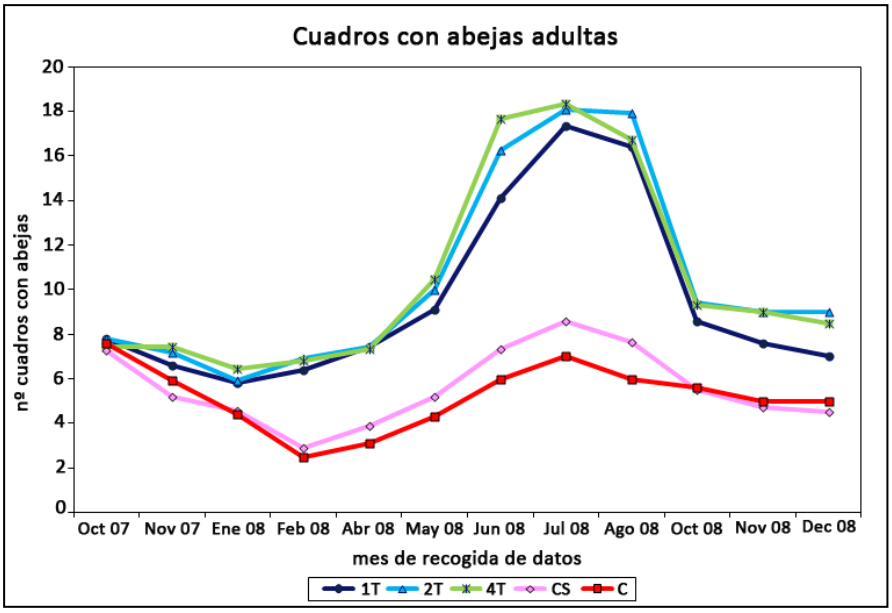



Figura A7. Número medio de cuadros con abejas adultas en cada grupo.

Al igual que en el caso de la población de abejas adultas, la cantidad de cría fue mayor en los grupos tratados en distintos puntos del ensayo.

En el inicio del ensayo (octubre del 2007), la cantidad de cría era similar entre los grupos (F = 0.6, *P* = 0.3). Sin embargo, los grupos 2T y 4T presentaron una mayor cantidad de celdillas de cría que los grupos CS y C en febrero (F = 4.6, *P* = 0.003) y abril (F = 5.4, *P* = 0.001; Figura A8).

En mayo del 2008, los grupos 2T y 4T mostraron mayor cantidad de cría que el grupo C (*P* = 0.03), y un mes más tarde (junio del 2008), únicamente el grupo 2T presentó más cría que los grupos control CS (*P* = 0.002) y C (*P* = 0.004).

Al final del ensayo, en octubre del 2008, el grupo 2T volvió a presentar una cantidad de cría significativamente mayor que los grupos CS ($P$ = 0.01) y C ($P$ = 0.007).

En las colonias tratadas, el número de celdillas de cría llegó a su máximo en junio, mientras que las colonias de los grupos CS y C lo alcanzaron en julio (Figura A8), si bien los niveles en los grupos no tratados fue muy inferior.

No se encontró una correlación significativa entre la proporción de abejas parasitadas y la cantidad de celdillas de cría ni en noviembre del 2007 (r = 0.28, $P$ = 0.11; coeficiente de correlación de Spearman) ni en julio del 2008 (r = - 0.19, $P$ = 0.27; coeficiente de correlación de Spearman).

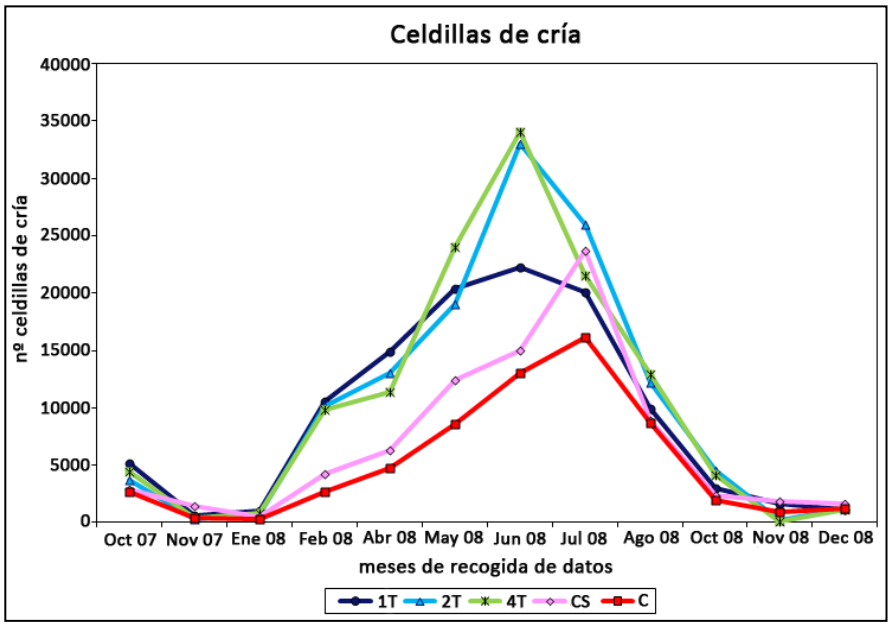



Figura A8. Número medio de celdillas de cría presente en cada grupo

Por otro lado, la proporción de celdillas de cría por cuadros de abejas adultas (NC/P) resultó ser significativamente mayor en julio en los grupos CS y C en comparación con los grupos tratados (F = 6.7, $P$ < 0.001; Figura A9). Sin embargo, en agosto únicamente el grupo CS presentó un ratio NC/P significativamente mayor que el mostrado por los grupos 1T ($P$ = 0.004), 2T ($P$ = 0.03) y 4T ($P$ = 0.006).

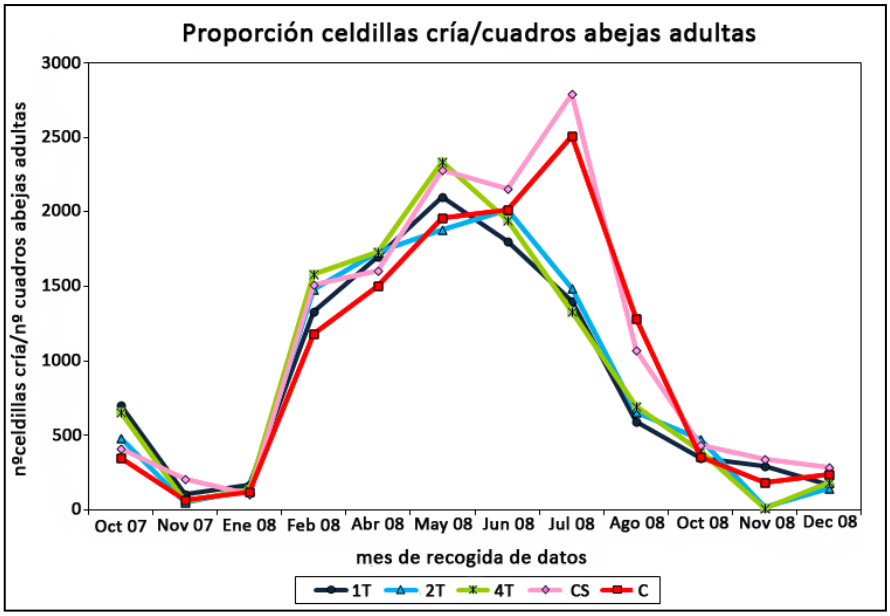



Figura A9. Proporción de celdillas de cría por cada cuadro con abejas adultas en cada grupo.

### 2.2.2. Producción de miel

Las colonias del grupo 4T fueron las que produjeron una mayor cantidad de miel (24.1 ± 7.3 kg miel/cololnia), aunque con un nivel muy similar al alcanzado por las del grupo 2T (21.2 ± 7.3 kg m/c; Figura A9a). Ambos grupos fueron significativamente más productivos ($P < 0.05$) que los grupos CS (11.5 ± 5.1 kg m/c) y C (8.9 ± 5.9 kg m/c).

Las colonias del grupo 1T produjeron menos miel (14.5 ± 12.1 kg m/c) que las de los otros grupos tratados, pero más que las de los grupos control (Figura A9a), aunque estas diferencias no fueron estadísticamente significativas ($P > 0.05$). En este grupo hubo una gran variabilidad en la producción de miel entre colonias.

Se encontró una correlación negativa entre el porcentaje de abejas parasitadas por *Nosema* en julio y la cantidad de miel producida ($r = - 0.51$, $P = 0.002$; coeficiente de correlación de Spearman).

Las colonias que murieron antes de la época de recolección (2 colonias del grupo 4T, 3 colonias del grupo CS y 3 colonias del grupo C) no fueron tenidas en cuenta al calcular la cantidad media de miel producida en cada uno de los grupos (Figura A9b).

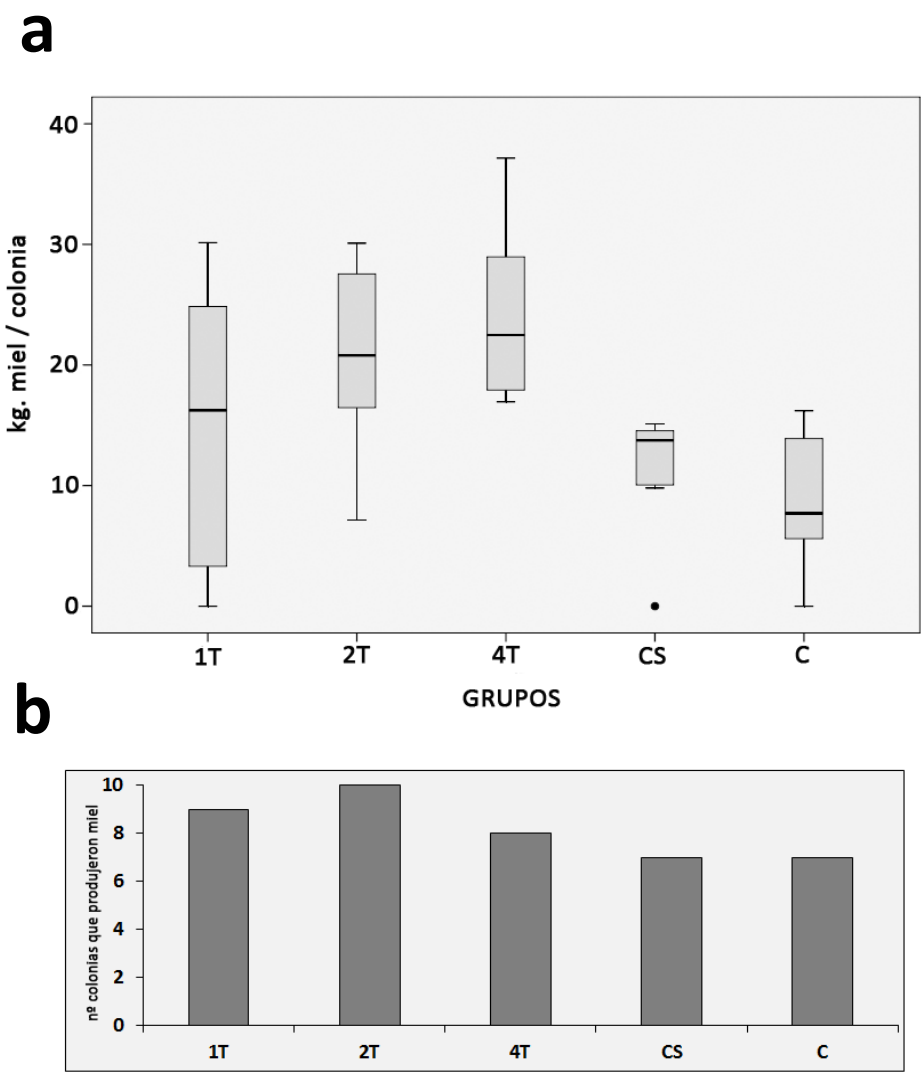



Figura A9. Producción de miel en las colonias estudiadas:

a: Media de miel producida (kg) por grupo en el periodo de recolección (Septiembre del 2008)

b: Número de colonias productivas en cada grupo en el periodo de recolección.

## 2.3. Reemplazo natural de la reina y mortalidad de las colonias

Únicamente 4 de las 50 colonias sufrieron un cambio de reina natural durante el estudio: 2 colonias del grupo 1T (colonias 1T-6 y 1T-8), 1 colonia del grupo 4T (4T-9) y una colonia del grupo C (C-7) en junio del 2008, mientras que la colonia C-3 reemplazó su reina en julio del 2008 (Figura A3).

La mayor viabilidad de colonias se observó en el grupo 2T, en el que no murió ninguna colonia. El resto de los grupos perdieron 1 (10%, 1T), 2 (20%, 4T) o 3 colonias (30%; CS y C) antes de la finalización del ensayo en diciembre del 2008 (Figura A3).

La forma de colapso fue en general por el desarrollo de enfermedades de la cría, como loque americana (1 colonia del grupo CS) o ascosferosis (1 colonia del grupo 4T), por ausencia de reina viable o cría zanganera (1 colonia del grupo 4T, 1 del grupo 1T y 2 colonias del grupo CS), y por despoblamiento (2 colonias del grupo CS y 1 del grupo C). En todas estas colonias que murieron no se detectó la presencia de abejas muertas o reptantes alrededor de las colmenas, y tampoco se observaron marcas fecales en las mismas.

En los análisis previos al colapso, todas las colonias muertas contenían un porcentaje de abejas pecoreadoras infectadas por *N. ceranae* superior al 45% (rango 45-95%).

Durante el seguimiento que se realizó tras el ensayo (diciembre 2008-diciembre 2010) 2 colonias del grupo 1T murieron mostrando signos clínicos de loque americana, y 4 colonias del grupo CS y 3 del grupo C murieron por despoblamiento (Figura A3).

Por tanto, en Diciembre del 2010, el porcentaje de colonias vivas era del 70% en el grupo 1T, 100% en el grupo 2T, 80% en el grupo 4T, 30% en el grupo CS y 40% en el grupo C.

# Trabajo Experimental 2

## Evaluación de la eficacia de posibles agentes terapéuticos para tratar las nosemosis en las colonias de abejas melíferas

*Trabajo experimental no publicado*

*En este ensayo se evalúa la eficacia de tres posibles agentes terapéuticos para el tratamiento de las nosemosis en las colonias de abejas melíferas, comparando su acción con la mostrada por la fumagilina, cuya eficacia ha sido anteriormente probada en el control de las nosemosis. Los productos evaluados fueron Nosestat® (m.a. yodo y ácido fórmico), Salicilato de Fenilo (éster salicílico del fenol) y Vitafeed Gold® (*Beta vulgaris*).*

*Ninguno de los productos evaluados mostró eficacia contra las nosemosis en nuestras condiciones experimentales, ya que las colonias continuaron presentando infección por* Nosema *spp. tras la aplicación de los mismos. La falta de un consumo adecuado de las distintas dosis suministradas de estos productos, sobre todo en otoño, pudieron haber influido en esta ausencia de eficacia, por lo que los estudios con este tipo de productos potencialmente terapéuticos deberían continuar, evaluando la metodología óptima de aplicación.*

***PRESENTACIÓN EN CONGRESO:***

*Botías C., Martín-Hernández R., Meana A., Higes M. Screening of possible treatments to control* Nosema ceranae *infection in honey bee (*Apis mellifera iberiensis*) colonies. Conferencia Norteamericana de Apicultura, 10-14 Enero 2012, Las Vegas (EEUU).*

# 1. Material y métodos

## 1.1. Colonias de abejas

En uno de los colmenares experimentales del Centro Apícola Regional (CAR) se instalaron cincuenta colonias de *Apis mellifera iberiensis* con el fin de examinar la eficacia de tres agentes potencialmente terapéuticos para las nosemosis (Nosestat®, Salicilato de fenilo, Vitafeed Gold®), y comparar su acción con la de la fumagilina (Fumidil-B®; Higes y col., 2011).

A estas 50 colonias se les hizo un seguimiento mensual desde noviembre del 2008 hasta octubre del 2009. En junio del 2008, cuando estas colonias fueron instaladas en los colmenares del CAR, Al inicio del estudio, todas las colonias eran negativas a *Nosema* spp., *Varroa destructor*, loque americana, loque europea y ascosferosis, y presentaban una población homogénea en cuanto a cantidad de población de abejas adultas, área de cría y alimento almacenado. Las enfermedades de la cría (loque americana, loque europea y ascosferosis) fueron evaluadas por medio de la observación de una ausencia de los signos clínicos característicos de estas patologías, mientras que la posible presencia de *V. destructor* se examinó en ácaros en adultos y cría, y la de *Nosema* spp. fue analizada por medio de los métodos descritos en el Artículo 1 de esta memoria (Martín-Hernández y col. ,2011).

Los colmenares utilizados para este ensayo se encontraban próximos a otros que contenían colonias parasitadas por *Nosema* spp., actuando como fuente de infección para las nuevas colonias instaladas.

La parasitación por *V. destructor* se controló dos veces al año mediante la utilización de Apivar® (m.a. amitraz) para evitar las repercusiones negativas de este parásito sobre el estado sanitario de las colonias en estudio.

## 1.2. Condiciones experimentales del ensayo

En noviembre del 2008 se analizaron las 50 colonias del ensayo y todas eran positivas a *N. ceranae*. Posteriormente, éstas fueron distribuidas al azar en cinco grupos, conteniendo 10 colonias de abejas cada uno de ellos. En cada uno de los grupos se aplicó un tratamiento diferente, tal y como muestra la tabla B1.

Cada una de las dosis de productos ensayados se administró en las colonias en bolsas de plástico para uso alimentario, depositándose las mismas sobre la cámara de cría, con excepción del producto VitaFeed Gold®, el cual fue administrado por goteo en las correspondientes colonias, siguiendo las recomendaciones del fabricante.

En el caso de las dosis aplicadas en bolsas de plástico (Nosestat®, Salicilato de Fenilo y Fumidil-B®), éstas se retiraron en el momento de aplicación de la siguiente dosis en caso de no haber sido consumidas completamente, procediéndose en estos casos a medir el volumen de producto remanente en la bolsa. En cuanto a Vitafeed Gold®, el análisis sobre su consumo no fue posible debido a la metodología de aplicación utilizada.

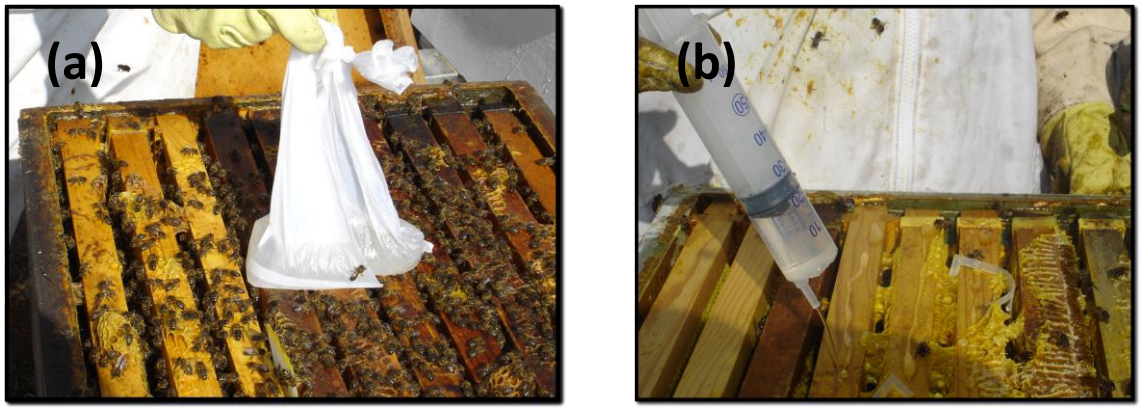



Figura B1. Aplicación de tratamientos por medio de bolsas de plástico (a) o por goteo (b).

| 50 COLONIAS ESTUDIADAS | | | | | | | |
|---|---|---|---|---|---|---|---|
| GRUPO | Nº COLONIAS | PRODUCTO APLICADO | POSOLOGÍA | | | | MOMENTO APLICACIÓN |
| | | | CONCENTRACIÓN MEZCLA | VOLUMEN DOSIS | FRECUENCIA | VOLUMEN TOTAL | |
| FUM | 10 | FUMIDIL-B® (Fumagilina 2%) | 6 g / 1 L jarabe | 250 ml mezcla/dosis | 1 dosis a la semana durante cuatro semanas seguidas | 1 L / colonia | OTOÑO 08 / PRIMAVERA 09 |
| NOS | 10 | NOSESTAT® (Yodo – Ácido fórmico) | 3.75 ml / 1 L jarabe | 250 ml mezcla/dosis | 3 dosis repartidas en tres días alternativos | 750 ml / colonia | OTOÑO 08 / PRIMAVERA 09 |
| SAL | 10 | SALICILATO DE FENILO | 18 g / 1 L jarabe | 250 ml. mezcla/dosis | 1 dosis a la semana durante cuatro semanas seguidas | 1 L / colonia | OTOÑO 08 / PRIMAVERA 09 |
| VIT | 10 | VITAFEED GOLD® (extracto de *Beta vulgaris*) | 100 g / 1 L jarabe | 100 ml. mezcla/dosis | 5 dosis, aplicando cada una con dos días de intervalo entre ellas | 500 ml / colonia | OTOÑO 08 / PRIMAVERA 09 |
| C | 10 | SIROPE | Azúcar - Agua 1:1 | 250 ml. sirope/dosis | 1 dosis a la semana durante cuatro semanas seguidas | 1 L / colonia | OTOÑO 08 / PRIMAVERA 09 |

Tabla B1. Grupos de colonias del estudio, productos y posología utilizada en los momentos de aplicación.

## 1.3. Seguimiento de la infección por *Nosema* spp.

Con el fin de evaluar la eficacia de los productos aplicados para el control de las nosemosis, se recogió una muestra de abejas pecoreadoras (n ≥ 50 abejas) antes de aplicar los tratamientos y una vez consumida la última dosis de los mismos, analizando la presencia de *Nosema* spp. en dichas muestras siguiendo el protocolo descrito en el Artículo 1 de esta memoria (Martín-Hernández y col., 2012). Esta operación se realizó tanto en otoño 2008 como en la de primavera 2009.

## 1.4. Seguimiento de los parámetros de vitalidad

A lo largo del ensayo, se evaluó la población de abejas adultas, la cantidad de cría operculada y la producción de miel en las colonias ya que estudios previos habían demostrado que la infección por *Nosema* sp. se puede reconocer en las colonias a través de estos signos clínicos (Fries, 1988; Yücel y Dogaroglu, 2005; Higes y col., 2008a). Esta evaluación se realizó siguiendo el protocolo descrito en el Trabajo Experimental 1 de esta memoria.

Tanto la cantidad de población de abejas adultas como la de cría operculada fue cuantificada una vez al mes desde diciembre del 2008 hasta octubre del 2009 (excepto en agosto del 2009), realizando un total de 10 mediciones. A su vez, la cantidad de miel producida por cada una de las colonias fue medida al final de la temporada apícola (septiembre 2009).

## 1.5. Análisis estadístico

Las variables dependientes estudiadas fueron la población de abejas adultas y el número de celdillas de cría en cada uno de los grupos de colonias, tomando cada tratamiento como factor fijo, mientras que el tiempo se tomó como factor repetido.

Debido a la variación encontrada con respecto al tiempo de las variables evaluadas (población y cría), sus valores medios se compararon entre los grupos a lo

largo de los distintos puntos de tiempo del ensayo utilizando un análisis de varianza (ANOVA) en una dirección. Cuando se encontraron diferencias significativas entre los distintos grupos del ensayo, los análisis de varianza fueron seguidos de un test post hoc Bonferroni o Tamhane dependiendo de la homogeneidad de varianza en cada caso (determinado utilizando el test de Levene).

En cuanto a la producción de miel, al tratarse de un tamaño muestral pequeño y de una distribución no normal de los datos, se utilizó una prueba no paramétrica (Kruskal-Wallis) para comparar las medias de cada uno de los grupos, utilizando el test de Mann-Whitney para realizar las comparaciones dos a dos entre grupos. Las diferencias entre grupos fueron consideradas significativas cuando $\alpha \leq 0,05$. Los análisis fueron realizados utilizando el programa informático SPSS 18.0.

# 2. Resultados

## 2.1. Seguimiento de la infección por *Nosema* spp.

Tras el análisis de las muestras en noviembre 2008 (inicio de los ensayos) todas las colmenas del estudio (N=50) se habían infectado de manera natural con *N. ceranae*.

Una vez aplicados los diferentes productos en otoño del 2008, todas las colonias del ensayo permanecieron parasitadas por *N. ceranae*. Por otro lado, se observó un bajo consumo de los tratamientos aplicados, excepto en el caso de VitaFeed Gold®, cuyo consumo no pudo ser evaluado ya que la aplicación de éste se realizó mediante goteo (Tabla B2).

| GRUPO | CONSUMO (% dosis aplicada) | |
|---|---|---|
| | OTOÑO ´08 | PRIMAV. ´09 |
| FUM | 52.2% | 100% |
| NOS | 36.7% | 100% |
| SAL | 38.75% | 77.5% |
| C | 70% | 100% |

Tabla B2. Consumo medio (%) de los agentes potencialmente terapéuticos administrados a las colonias del ensayo.

Las colonias consumieron mejor los tratamientos aplicados en primavera del 2009 (Tabla B2) sin embargo los resultados mostraron una vez más la presencia de *N. ceranae* en todas las colonias del estudio tras la aplicación de los mismos, apareciendo además *N. apis en* co-infección con *N. ceranae* en una colonia del grupo NOS (Nosestat®), en otra del grupo SAL (Salicilato de Fenilo) y en otra del grupo VIT (Vitafeed Gold®). Únicamente en el caso de las colonias tratadas con fumagilina se apreció que el 30% de las mismas estaban libres de infección tras la aplicación del tratamiento (Figura B2).

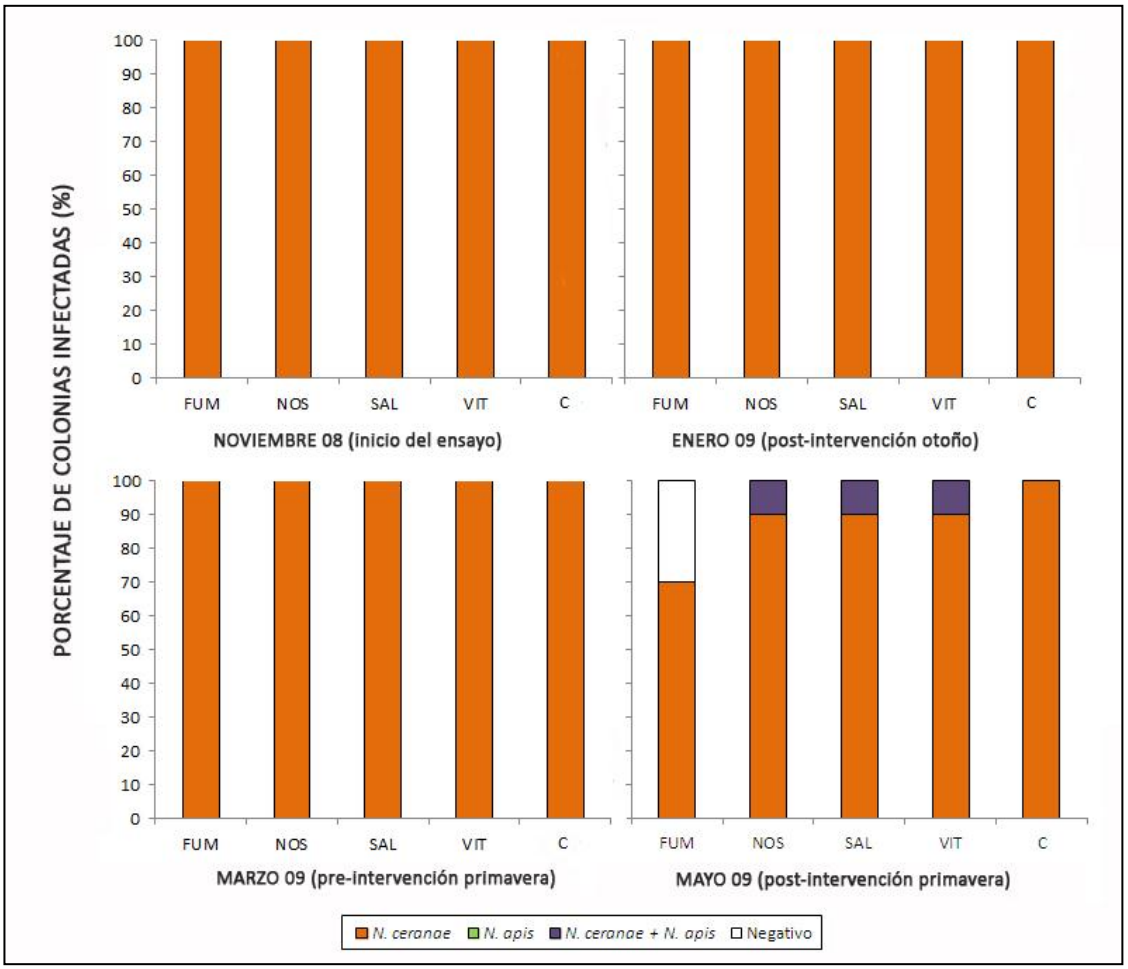



Figura B2. Porcentaje de colonias infectadas en cada uno de los grupos experimentales, antes y después de intervenir en las mismas tanto en otoño 08 como en primavera 2009.

## 2.2. Seguimiento de los parámetros de vitalidad

La población de abejas adultas en los distintos grupos se mantuvo similar a lo largo del ensayo, no apareciendo diferencias significativas entre las valores medios de población, excepto en Junio 2009, cuando las colonias del grupo FUM mostraron una población significativamente mayor que la del grupo C (ANOVA, $p$ = 0,037; Figura B3).

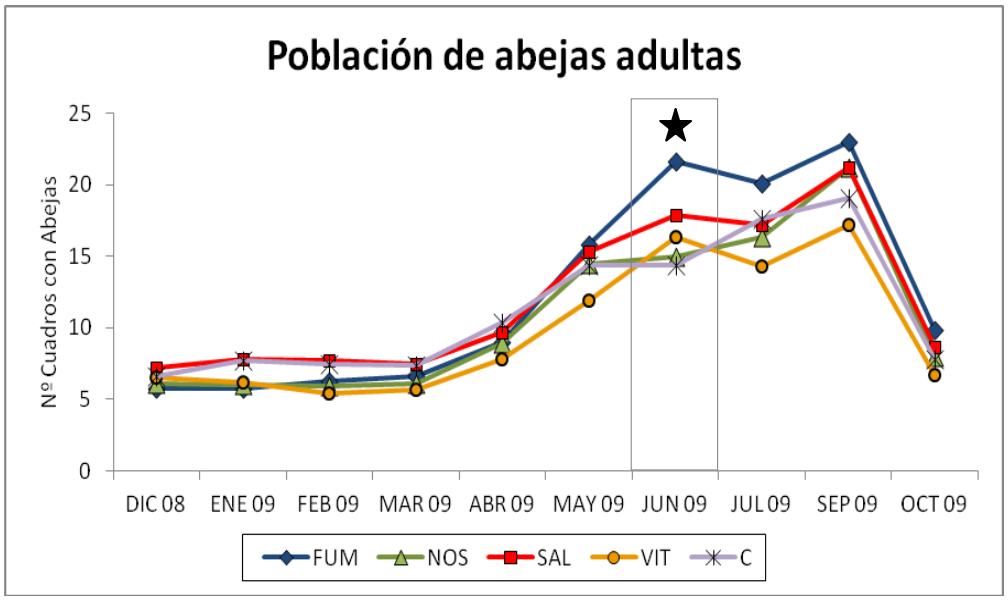



Figura B3. Cantidad media de cuadros de abejas adultas por grupo (la estrella indica diferencias significativas entre grupos).

En el caso del número de celdillas con cría, no se encontraron diferencias significativas entre los grupos a lo largo del ensayo ($p > 0,05$; Figura B4).

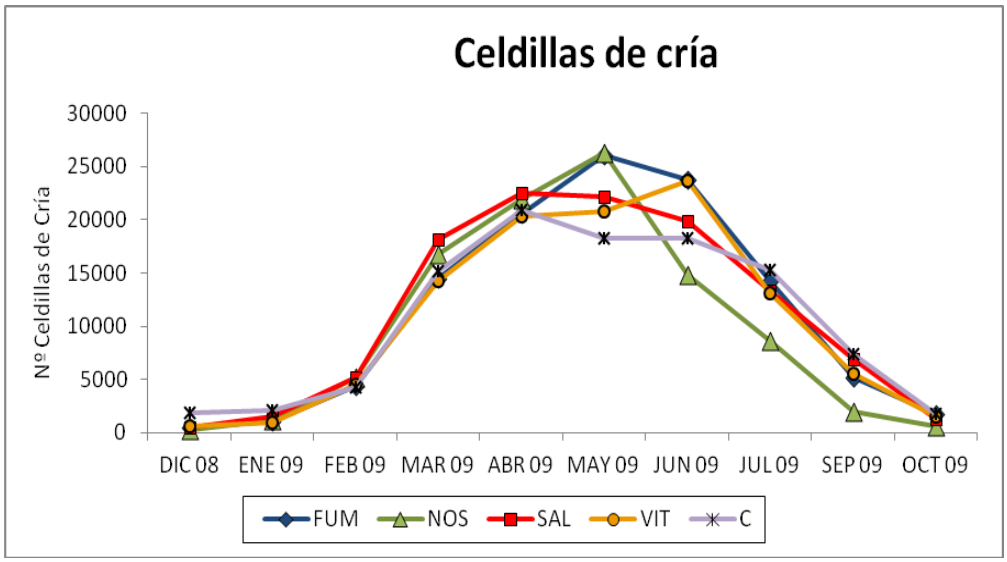



Figura B4. Número medio de celdillas de cría en cada uno de los grupos del estudio a lo largo del tiempo.

Con respecto a la producción de miel, el grupo FUM produjo más miel (18,7 ± 5,6 kg. por colonia; Figura B5) que el resto de los grupos, siendo estas diferencias estadísticamente significativas al compararlo con los grupos VIT (11,5 ± 6,5 kg. por colonia; test de Mann-Whitney, U=10, *p*=0.037) y C (12,3 ± 7,9 kg. por colonia; Mann-Whitney, U=9, *p*=0.011). Las colonias del grupo NOS (14,4 ± 5,3kg. por colonia) y del grupo SAL (14,02 ± 7,7 kg. por colonia) no mostraron diferencias significativas con respecto al resto de grupos del ensayo.

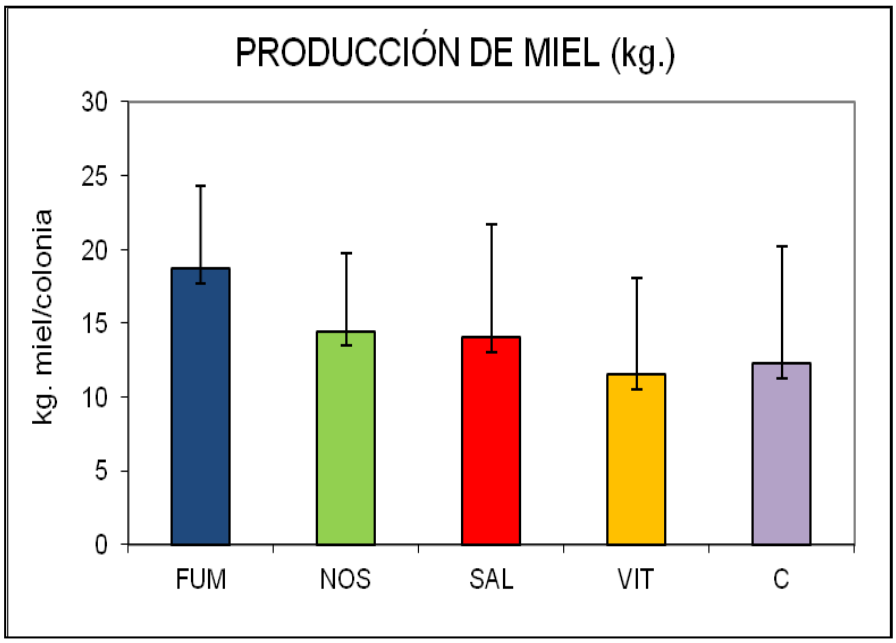



Figura B5. Cantidad media de miel (kg.) producida por colonia en cada uno de los grupos experimentales.

# DISCUSIÓN GENERAL

La abeja melífera es uno de los insectos más provechosos para los ecosistemas y por extensión para la humanidad, dada la importancia de la producción de miel y otros productos de la colmena (jalea real, cera, propóleos, polen, veneno), así como por su papel crucial en la polinización de cultivos y plantas silvestres a lo largo del planeta (Bradbear, 2009). Por ello, el mantenimiento de las poblaciones de abejas melíferas en condiciones sanitarias adecuadas es esencial, ya que una pérdida de las mismas podría causar graves problemas tanto para los humanos como para la biodiversidad (Brown y Paxton, 2009).

La nosemosis tipo C se considera como una de las enfermedades más dañinas para las poblaciones de abejas y la apicultura (Higes y col., 2010; Heintz y col., 2011) junto con la varroosis provocada por el ácaro ectoparásito *V. destructor* (Le Conte y col., 2010), siendo además las enfermedades más prevalentes en las colonias de abejas.

En cuanto a la nosemosis causada por *N. apis* (nosemosis tipo A), se trata de una enfermedad ampliamente distribuida en las poblaciones de abejas de todo el mundo desde hace décadas, y su acción patógena ha sido profusamente estudiada (Farrar, 1947; Moeller, 1962; Kauffeld y col., 1972; Fries, 1988; OIE, 2008). A pesar de ello, en raras ocasiones ha sido considerada como una enfermedad grave para la colonia por parte de la comunidad científica y los profesionales de la apicultura (Topolska y Hartwig, 2005; Hornitzky, 2008). Esto se debe a que su prevalencia suele ser baja (OIE, 2008; Martín-Hernández y col., 2012) y los casos agudos de esta enfermedad son poco frecuentes, llegándose en pocas ocasiones al colapso final de las colonias infectadas (White, 1919; Bailey, 1981; Gisder y col., 2010; Moritz y col., 2010). Esta situación hace pensar que dicho parásito podría haber sufrido una co-evolución durante décadas con su hospedador original, lo que habría dado lugar a un equilibrio en la relación entre ambos organismos, permitiendo así la existencia de una forma crónica pero no mortal de dicha enfermedad. Por otro lado, a partir del año 2005, en el cual se descubrió que *N. ceranae* también era capaz de causar infección en la abeja melífera europea (*A. mellifera*; Higes y col., 2005, 2006), diversos estudios han indicado que la infección provocada por este microsporidio es más prevalente en la colonias de abejas que la provocada por *N. apis* (Chen y col., 2008; Tapaszti y col.,

2010; Stevanovic y col., 2011; Soroker y col., 2011; Traver y Fell, 2011), alcanzando niveles de epidemia en poblaciones de colonias de diversas regiones geográficas (revisado por Higes y col., 2010). Además, ciertos trabajos han puesto de manifiesto las diferencias en la acción patógena de estos dos microsporidios, mostrando *N. ceranae* una mayor virulencia en los distintos ensayos experimentales realizados (Paxton y col., 2007; Antúnez y col., 2009; Martín-Hernández y col., 2011), así como una mayor capacidad para causar el debilitamiento y colapso de las colonias de abejas en ciertas condiciones. De hecho, diversos estudios han sugerido que la nosemosis tipo C podría ser un factor clave en el fenómeno de despoblamiento de las colonias de abejas experimentado en ciertas regiones (Higes y col., 2008a, 2009b; Bacandritsos y col., 2010; Soroker y col., 2010; Bromenshenk y col., 2011; Hatjina y col., 2011; Garrido Bailón, 2012). Por tanto, a pesar de que ambos microsporidios se han encuadrado hasta el momento en el mismo género, sorprende que tanto desde un punto de vista epidemiológico como patogénico se detecten diferencias importantes, lo que origina finalmente efectos muy diferentes sobre la colonia de abejas.

La mayor prevalencia presentada por *N. ceranae* con respecto a *N.* apis en todo el planeta ha llevado a la comunidad científica a plantearse la hipótesis de que *N. ceranae* podría estar desplazando a su congénere *N. apis* en su nicho ecológico compartido, las poblaciones de abejas melíferas (Klee y col., 2007; Chen y col., 2009a; Cornmann y col., 2009; Yoshiyama y Kimura, 2011). En el Artículo 1 de esta memoria se muestra que en ninguna de las escalas estudiadas (país, apiario, colonia) parece estar ocurriendo este reemplazo, coincidiendo con los resultados observados por otros grupos de investigación (Budge, 2010; Genersch y col., 2010; Gisder y col., 2010). Sin embargo, sí se ha encontrado una mayor prevalencia de *N. ceranae* en todas las escalas estudiadas, además de una presencia de ésta en las colonias durante todo el año, mientras que la prevalencia de *N. apis* (mucho menor) siguió un patrón epidemiológico clásico (mayor en primavera y otoño; Borchert, 1928; Bailey, 1955), y con valores similares a los descritos históricamente en nuestro país (Gómez Pajuelo y Fernández Arroyo, 1979; Orantes Bermejo y García Fernández, 1997) y en otras latitudes (Dr. R. Cramer JR., comunicación personal; Klee y col., 2007; Giersch y col., 2009; Runckel y col., 2011). Este fenómeno podría indicar que *N. ceranae* presenta

alguna ventaja desde el punto de vista epidemiológico con respecto a *N. apis* en condiciones de campo, permitiéndole una mayor dispersión y persistencia en ciertas condiciones climáticas en las poblaciones de *A. mellifera* tanto dentro de la colonia como dentro de un apiario, e incluso a nivel nacional.

A su vez, los resultados mostrados en el Artículo 2 sugieren que la entrada de *N. ceranae* en nuestro país sería un fenómeno relativamente reciente y su prevalencia habría crecido de manera significativa en la última década en las regiones con una mayor actividad apícola en nuestro país. Estos datos confirmarían los resultados previos sobre el aumento en la detección de nosemosis en nuestro país debida a la colonización de *A. mellifera* por parte de *N. ceranae* (Martín-Hernández y col., 2007), situación que no se corresponde con lo observado en países como Alemania o Reino Unido (Budge, 2010; Gisder y col., 2010), donde *N. apis* parece ser la especie más frecuente. Estas diferencias observadas en distintos puntos del planeta podrían estar relacionadas con factores climáticos (Giersch y col., 2009; Gisder y col., 2010; Martín-Hernández y col., 2012), diferencias en el manejo de las colonias (Botías y col., 2012a), en la metodología de muestreo y análisis de las muestras (Bourgeois y col., 2011; Botías y col., 2012c), o por una presencia de haplotipos de *Nosema* de patogenicidad dispar (Williams y col., 2010; Medici y col., 2011), aunque estas conjeturas deberían ser confirmadas por estudios futuros.

Por otro lado, los resultados del Artículo 3 apoyan la hipótesis del origen asiático del hospedador original de *N. ceranae*, y apuntan hacia una entrada de este microsporidio en las poblaciones occidentales de abejas melíferas siguiendo rutas similares a las que llevaron al ácaro *V. destructor*, originalmente asociado con *A. cerana*, a convertirse en el parásito más extendido en las colonias de *A. mellifera*. De este modo, tal y como se describe en este estudio, uno de los factores participantes en la dispersión global de *N. ceranae* podría haber sido la invasión de especies portadoras del patógeno (*e.g. A. cerana*) en territorios con poblaciones de *A. mellifera* sin previo contacto con dicho microsporidio.

En esta memoria (Artículos 3 y 4) también se muestra la mayor capacidad de *N. ceranae* para infectar a un mayor rango de hospedadores que *N. apis*, lo cual podría

indicar diferencias en sus mecanismos de infección, así como una mayor valencia ecológica en el caso de *N. ceranae*. Mientras que *N. apis* ha sido detectada únicamente en poblaciones de abejas de *A. mellifera* y menos frecuentemente en poblaciones de *A. cerana* (Chen y col., 2009a), *N. ceranae* se ha detectado hasta el momento en *A. mellifera*, en las especies de abejas asiáticas *A. cerana*, *A. florea*, *A. dorsata* (Fries y col., 1996; Chaimanee y col., 2010; Suwannapong y col., 2010) y *A. koschevnikovi* (Botías y col., 2012d), así como en varias especies de abejorros del género *Bombus* en Argentina y en China (Plischuk y col., 2010; Li y col., 2012), lo cual demuestra la mayor capacidad de este microsporidio para infectar a diversos hospedadores y así explotar un mayor número de hábitats. La especificidad es una característica común en los microsporidios y su rango de hospedadores suele ser muy limitado (Solter y Becnel 2003; Solter y col., 2005). Sin embargo, *N. ceranae* es capaz de transmitirse con éxito entre distintos hospedadores para expandir su rango y establecer de este modo la infección en nuevas especies de himenópteros polinizadores.

La alta patogenicidad de *N. ceranae* en *A. mellifera* ha sido observada en distintos estudios (Paxton et al., 2007, Higes et al., 2008; Dussaubat y col., 2009, 2012; Alaux y col., 2011; Martín-Hernández y col., 2011; Vidau y col., 2011), pero las repercusiones de su infección en otros hospedadores como *Bombus* spp., *A. dorsata* o *A. koschevnikovi* no es conocida hasta el momento. Además de en *A. cerana* (Fries y col., 1996) y en *A. mellifera* (Higes y col., 2007; Antúnez y col., 2009; Dussaubat y col., 2009; Alaux y col., 2010), únicamente en el caso de la especie asiática *A. florea* ha sido estudiada la acción patógena de *N. ceranae* (Suwannapong y col., 2010), encontrándose una mayor tasa de mortalidad y una disminución en la producción proteica de las glándulas hipofaríngeas de las abejas infectadas. Las consecuencias en la supervivencia de aquellos polinizadores susceptibles a la infección con *N. ceranae* deberían ser evaluadas para establecer un rápido y eficaz control de una posible enfermedad con el fin de asegurar la conservación de estas especies.

Además, en el Artículo 5 se aportan por primera vez evidencias sobre la presencia de *N. ceranae* en las poblaciones de abejas melíferas (*A. mellifera intermissa*) en el continente africano, demostrándose la aparente omnipresencia y rápida dispersión de este patógeno en las poblaciones de *A. mellifera*. De hecho, *N. ceranae* se ha

encontrado infectando colonias de abejas en los cinco continentes habitados del mundo (Klee y col., 2007; Chen y col., 2008, 2009; Williams y col., 2008; Calderón y col., 2009; Giersch y col., 2009; Yoshiyama y Kimura, 2010), al igual que *N. apis* (Furgala y Munsen, 1990; Matheson, 1996), aunque aparentemente su propagación se habría producido en un periodo de tiempo relativamente más corto. Este fenómeno podría estar relacionado con la globalización en el comercio de material biológico y productos de la colmena que se ha producido de manera más generalizada en las últimas décadas, llevado a cabo sin una regularización específica que prevenga la dispersión de los patógenos de las abejas en la mayor parte de los países implicados.

Aunque ambas especies de *Nosema* infectan las células epiteliales del ventrículo de las abejas, su potencial biótico dentro de las células infectadas muestra diferencias, lo que podría justificar que las consecuencias patológicas de la infección para cada especie de microsporidio no sean las mismas incluso cuando se detecten recuentos de esporas similares. Según los resultados que se muestran en el Artículo 6, *N. ceranae* tiene una mejor adaptación para completar su ciclo biológico y muestra un mayor índice biótico a lo largo de un rango de temperaturas más amplio, lo que explicaría que *N. ceranae* sea más prevalente en climas templados que *N. apis* (Chauzat y col., 2007; Chen y col., 2010; Higes y col., 2009b; Tapaszti y col., 2009; Fries, 2010; Dainat y col., 2012; Martínez y col., 2012). Por otra parte, en las fases iniciales de la infección por *N. ceranae,* a 33°C, hay un mayor número de fases inmaduras (70%) que de esporas maduras. En *N. apis* a la misma temperatura, la proporción es igual entre fases maduras e inmaduras (50%). Además, el estudio presentado en el Artículo 7 sugiere que *N. ceranae,* a diferencia de *N. apis*, sería capaz de continuar su desarrollo y proliferación a temperaturas (37°C) más elevadas que las habituales en la cámara de cría (≈ 33°C), mientras que *N. apis* ve inhibido su desarrollo a dicha temperatura. Esta aparente euritermia, unida a una mayor resistencia a la desecación de *N. ceranae* (Fenoy y col., 2009), permitiría a este microsporidio continuar con su reproducción y dispersión, y por tanto, le concedería una mayor adaptación, en situaciones de calor extremo y sequía. Sin embargo, *N. apis* parece soportar mejor las bajas temperaturas (Forsgren y Fries, 2010; Gisder y col., 2010), conservando un índice de infectividad mayor que las esporas de *N. ceranae* tras la congelación. En este caso, este hecho

podría suponer una ventaja para *N. apis* en los climas muy fríos, ya que las esporas remanentes en el material apícola u otros fómites serían capaces de conservar su capacidad infectiva y esperar en estado latente al momento propicio para causar infección. Estas diferencias en los patrones de desarrollo dependientes de la temperatura podrían explicar la mayor prevalencia de *N. ceranae* en los pisos bioclimáticos más cálidos de nuestro país, y de *N. apis* en las áreas que presentan una época de heladas más larga (Martín-Hernández y col., 2012). Del mismo modo, este fenómeno podría estar relacionado con la mayor severidad mostrada por la nosemosis tipo C en las colonias de abejas de latitudes con climas más cálidos (Higes y col., 2008a, 2009b; Bacandritsos y col., 2010; Hatjina y col., 2011; Eischen y col., 2012), y la menor prevalencia encontrada en ciertos países que alcanzan temperaturas muy bajas en invierno (Budge, 2010; Fries, 2010; Gisder y col., 2010), aunque la implicación de los factores climáticos en los patrones epidemiológicos mostrados por estos microsporidios deberían ser evaluados con más detenimiento en futuros estudios.

Por otro lado, dentro de los posibles factores ambientales que intervienen en la propagación de *Nosema* se encuentran los abejarucos (*Merops* spp.), pájaros insectívoros que podrían participar en la dispersión de esporas de *Nosema* en las áreas en las cuales éstos se alimentan y crían, tal y como sugieren los resultados del Artículo 8. A su vez, este ensayo mostró únicamente la presencia de *N. ceranae* en las egagrópilas de los abejarucos, lo cual podría ser un reflejo de la presencia mayoritaria de este microsporidio en las colonias de abejas en nuestras condiciones (Martín-Hernández y col., 2007). Un estudio posterior aporta evidencias sobre la capacidad de dispersión de *N. ceranae* a gran escala (escala continental) por parte de estos pájaros (Varela y col., 2010), lo cual apoya la hipótesis enunciada sobre el papel de los abejarucos como potenciales agentes de dispersión de las esporas de este microsporidio. Asimismo, este mismo estudio confirma la presencia dominante de *N. ceranae* con respecto a *N. apis* en las egagrópilas de abejarucos en distintos puntos del planeta, lo que puede estar relacionado con la mayor prevalencia de este microsporidio en la mayor parte de las poblaciones de abejas, o con una mayor resistencia de las esporas de *N. ceranae* a las distintas condiciones ambientales (Fenoy y col., 2009). Teniendo en cuenta que *Merops apiaster* es una de las 26 especies de

abejarucos que existen en el mundo, las cuales se presentan en África, Europa, Asia o Australia (Fry y col., 2000), es muy probable que la propagación de esporas de *Nosema* por parte de los abejarucos ocurra a escala continental por medio de distintas especies de pájaros insectívoros. Aunque los abejarucos no están presentes en América, sí existen otras especies de pájaros que se alimentan de abejas (e.g. familias Tyrannidae y Mimidae), que podrían haber contribuido en esta dispersión a gran escala de *N. ceranae* en las poblaciones de abejas de esta región. Los factores que puedan afectar a los movimientos de estos pájaros (*e.g.* carga en el uso del terreno y prácticas agrícolas, cambio climático; Kinzelbach y col., 1997) podrían por tanto influir también en la propagación de esta enfermedad. Otros fómites que hayan podido participar en la amplia dispersión actual de *N. ceranae* son las herramientas utilizadas como material apícola en el manejo de las colonias de abejas (Van der Zee y col., 2010), la jalea real (Cox-Foster y col., 2007), la miel (Giersch y col., 2009; Botías y col., 2012e) o los pólenes de diversos orígenes geográficos utilizados como alimento para polinizadores comerciales como los abejorros y las abejas melíferas (Higes y col., 2008b; H.V.W. Velthuis, comunicación personal). Hasta el momento, la presencia de *N. ceranae* ha sido demostrada en todos los fómites anteriormente citados, pero la infectividad de las esporas presentes en los mismos únicamente ha sido probada en el caso del polen corbicular (Higes y col., 2008b) y de las egagrópilas de abejaruco (Higes y col., 2008c). Por ello, sería necesario el estudio de la capacidad infectiva de las esporas presentes en otras matrices para establecer las medidas de control y profilaxis adecuadas. Del mismo modo, debería ser evaluado el posible impacto del comercio global de productos de la colmena en la rápida dispersión de *N. ceranae* por el mundo (Mutinelli, 2011).

La existencia de diversas y numerosas fuentes de infección y dispersión de las esporas de *Nosema* spp. incrementa la dificultad de control de estos patógenos y hace indispensable un estudio detallado de las medidas preventivas y de profilaxis necesarias para el manejo de las colonias. Un entendimiento de los procesos de comportamiento que definen la estructura de este grupo social podría ayudar a identificar las rutas de transmisión utilizadas por estos patógenos para extenderse (Naug, 2010; Smith, 2012), lo cual a su vez podría dar pautas sobre los caminos a seguir

en el manejo de esta estructura social como medida para la prevención y el control de las nosemosis. En el Artículo 9 se propone un mecanismo zootécnico (la renovación de la reina) como método para reducir la carga parasitaria de *Nosema* en las colonias de abejas infectadas y de este modo controlar la enfermedad. En efecto, se pudo apreciar que este método, al igual que lo conseguido con el tratamiento con fumagilina, llevó a un descenso en el porcentaje de parasitación en las colonias infectadas. Ante la ausencia de tratamientos para controlar estas enfermedades y sus efectos negativos para las colonias de abejas y, por extensión, para la apicultura, agricultura y la biodiversidad, los resultados obtenidos con el cambio de reina aportan de una alternativa práctica para los apicultores en el control de la infección por *Nosema* spp. Sin embargo, estudios futuros deberían abordar la práctica de esta técnica con la introducción de una reina ya fecundada para evitar el parón en el desarrollo normal de la colonia, lo cual mostró tener efectos dañinos sobre la densidad poblacional a corto plazo y en la producción de miel. La introducción de una reina recién emergida ya fecundada de manera artificial en las colonias a las que se retiró la reina antigua podría haber evitado este paréntesis en la producción de cría y renovación de población adulta, y como consecuencia, quizás la producción de miel no se habría visto tan mermada en comparación con las colmenas que conservaron su reina inicial.

Por otro lado, en el Trabajo Experimental 1 se confirmó la eficacia del tratamiento con fumagilina (Fumidil B®) para controlar la infección por *N. ceranae* y *N. apis* en las colonias de abejas a corto plazo, coincidiendo con lo mostrado por estudios previos (Gregorc y Sulimanovic, 1996; Higes y col., 2008a, 2011; Williams y col., 2008). Según los resultados obtenidos, la fumagilina es capaz de reducir la carga parasitaria dentro de las colonias de abejas, ya que aquéllas en las que se suministró este medicamento bajaron sus porcentajes de parasitación de manera significativa. Debido a que *N. ceranae* fue la especie dominante en las colonias infectadas a lo largo del estudio, los efectos negativos sobre las colonias de abejas observados y descritos en el mismo podrían ser principalmente atribuidos a la acción de este microsporidio. Así, en nuestras condiciones experimentales, las colonias en las que los porcentajes de abejas infectadas por *N. ceranae* se mantuvieron muy altos durante todo el ensayo mostraron una cantidad de población y de celdillas de cría significativamente menor en ciertos

puntos del ensayo. Asimismo, la producción de miel también se vio afectada por la parasitación de estos microsporidios, ya que las colonias en las que no se controló la enfermedad presentaron niveles de producción de miel inferiores. Estos efectos sobre los parámetros de vitalidad se vieron confirmados al encontrar una correlación negativa entre el porcentaje de abejas infectadas por *N. ceranae* en verano y la cantidad de población y de miel producida en las colonias. Por tanto, teniendo en cuenta que la población de abejas adultas, la cantidad de cría operculada y el total de miel producida por una colonia se consideran como indicadores adecuados para estimar el estado sanitario de las colonias de abejas (Woyke, 1984; Meikle y col., 2008), los resultados de este estudio demuestran que estos microsporidios producen efectos muy dañinos en las colonias de abejas, tal y como se ha sugerido previamente (Moeller, 1978; Fries, 1984; Higes y col., 2008a, 2009b; Soroker y col., 2011; Botías y col., 2012a; Eischen y col., 2012). Sin embargo, la producción de cría no mostró esta correlación con los niveles de infección, aunque las colonias con un porcentaje de infección menor tuvieron significativamente más celdillas de cría que las colonias de los grupos altamente infectados en distintos puntos del ensayo, como ya se ha comentado anteriormente y coincidiendo con resultados de otros estudios (Moeller, 1962; Yücel y Dogaroglu, 2005; Higes y col., 2008a). Asimismo, la producción de cría fue más lenta en los grupos no tratados, que llegaron a su máximo en Julio, mientras que los grupos tratados comenzaron a crecer más temprano y llegaron a su máximo un mes antes (junio).

El tamaño de la población de abejas adultas y la producción de cría están correlacionados (Farrar, 1944; Moeller, 1958; Free y Racey, 1968; Soroker y col., 2011), y según estudios previos, las abejas infectadas por *N. ceranae* pueden sufrir un acortamiento en su esperanza de vida (Higes y col., 2007; Paxton y col., 2007; Martín-Hernández y col., 2011). Por tanto, las poblaciones más reducidas observadas en las colonias altamente infectadas por *Nosema* spp. podría ser el reflejo de una combinación de la menor producción de cría y un incremento en la mortalidad de las abejas pecoreadoras en esas colonias. Además, las tareas de termorregulación y de producción de alimento para las larvas podrían verse negativamente influidas en las

colonias infectadas con poblaciones reducidas (Wang y Moeller, 1970), dando lugar a una eficiencia reducida en la cría de larvas (Oldroy, 2007; Medrzycki y col., 2010).

Por otro lado, las colonias con altos niveles de infección por *Nosema* spp. a lo largo del estudio presentaron más celdillas de cría por cada cuadro de abejas adultas durante los meses de verano (julio y agosto) con respecto al resto de colonias. Según lo indicado por estudios anteriores, a medida que las colonias crecen se da una disminución en el ratio de cría-abejas (Farrar, 1932; Moeller, 1961; Free y Racey, 1968), tal y como se observó en la evolución presentada por las colonias con un bajo nivel de infección de este ensayo (grupos tratados). Sin embargo, las colonias altamente infectadas (grupos control) no crecieron al mismo nivel que las colonias tratadas, y no experimentaron una reducción similar en esa proporción de cría y población de abejas. El mayor ratio de cría-abejas presentado por las colonias altamente infectadas podría ser el resultado de un sobre-esfuerzo realizado por la colonia en un intento de reemplazar a las abejas obreras muertas debido a la infección por *Nosema* spp. (Farrar, 1947; Higes y col., 2008a), aunque para corroborar esta hipótesis serían necesarios más estudios.

Además, algunas de las colonias que no recibieron tratamiento para controlar la infección por *Nosema* spp. colapsaron sin detectarse abejas en el interior de las colmenas, coincidiendo con los signos descritos en un estudio previo (Higes y col., 2008a). A su vez, desde el punto de vista productivo, la acción patógena de *N. ceranae* podría acarrear serias consecuencias para la rentabilidad de la actividad apícola, ya que las colonias altamente infectadas por este microsporidio mostraron una producción significativamente menor que las colonias en las cuales se controló la enfermedad. Con respecto a los servicios de polinización, éste también podría verse afectado teniendo en cuenta que tanto la recolección de néctar como la de polen están negativamente correlacionadas con el estado sanitario de la colonia (Farrar, 1947; Fries y col., 1984, 1988; Szabo y Lefkovitch 1989; Anderson y Giacon, 1992; Schmid-Hempel y col., 1993), y las abejas pertenecientes a colonias menos pobladas, como son aquellas infectadas por *N. ceranae*, tienden a visitar una menor cantidad de flores por trayecto (Wolf y Schmid-Hempel, 1990). Por otra parte, las abejas pecoreadoras infectadas por *Nosema* ven limitada su capacidad de vuelo (Krajl y Fuchs,

2010; Mayack y Naug, 2010), lo cual, junto con todo lo anteriormente citado podría influir de manera negativa en el potencial polinizador de las colonias de abejas. Como ejemplo, en Estados Unidos, país en el cual sólo la industria de la almendra gana 2 billones de dólares anuales, se estima que esta actividad depende de la polinización de alrededor de 1 millón de colonias de abejas (Ratnieks y Carreck, 2010), y por lo tanto, en este caso concreto, un colapso masivo o reducción en la actividad polinizadora de colonias de abejas debido a la acción patógena de *N. ceranae* podría acarrear graves consecuencias económicas para este sector (Heintz y col., 2011). En cuanto al posible impacto sobre la biodiversidad, éste es incalculable, ya que muchas plantas silvestres, sobre todo en las áreas en las que *A. mellifera* es una especie nativa (*e.g.* Europa, África), dependen de la asociación mutualista entre sus flores y estas abejas para su reproducción, y por tanto, para su perpetuación como especies (Fontaine y col., 2006; Potts y col., 2010).

Una vez detectados los signos clínicos y sub-clínicos anteriormente descritos para la nosemosis tipo C (descenso en la densidad poblacional, en la cantidad de cría, en la producción de miel, aumento en el ratio de cría-abejas y aumento en la tasa de mortalidad de las colonias), se puede afirmar que *N. ceranae* no es sólo un agente patógeno para las abejas individualmente, sino también para la colonia de abejas. En esta enfermedad, distintos factores relacionados con el hospedador, en este caso la colonia de abejas, tendrán una influencia clave en el desarrollo y evolución de la enfermedad, como por ejemplo la "calidad" de la reina, el comportamiento higiénico y vitalidad de la estirpe de abejas que forman la colonia, la respuesta de su sistema inmune ante la infección (Huang y col., 2012), su tendencia al desarrollo de una inmunidad social (Evans y Spivak, 2010; Le Conte y col., 2011) o sus hábitos de pillaje y deriva. Ciertos factores externos a la colonia, como son las técnicas apícolas utilizadas por el apicultor o las condiciones climáticas del medio en que se desarrollan las colonias, también pueden tener un impacto en el desarrollo de una forma más o menos virulenta de esta enfermedad en las colonias. Asimismo, la virulencia de la cepa o cepas de este microporidio responsables de la infección en cada colonia también se ha propuesto como un factor que podría tener un impacto en el desarrollo de la enfermedad en la colonia (Williams y col., 2008; Medici y col., 2012), aunque éste

debería ser estudiado en base a marcadores moleculares que presenten una menor variabilidad en sus secuencias que los utilizados hasta el momento (*i.e.* gen 16S del ARN ribosómico) para poder establecer relaciones filogenéticas fiables entre los distintos haplotipos existentes de este microsporidio (Sagastume y col., 2010).

Aparte de los efectos negativos causados directamente por *N. ceranae* en las colonias de abejas, la interacción con otros factores como los pesticidas (Alaux y col., 2010; Vidau y col., 2011), y los virus (Bacandritsos y col., 2010; Bromenshenk y col., 2010) también han mostrado ser letales para las abejas. En este sentido, un reciente estudio ha descrito el incremento en las probabilidades de desarrollar enfermedades de la cría como la ascosferosis provocada por el hongo *Ascosphaera apis* (Hedtke y col., 2011) en aquellas colonias afectadas por *N. ceranae*. Esta observación fue corroborada por los resultados del Trabajo Experimental 1, ya que las colonias que mantuvieron una infección con *N. ceranae* a largo plazo mostraron una tendencia a desarrollar otras patologías como la mencionada ascosferosis, o incluso loque americana, enfermedad bacteriana de la cría causada por *Paenibacillus larvae*.

Con respecto al medicamento veterinario utilizado (Fumidil-B®), se pudo apreciar que éste carece de una eficacia a largo plazo, ya que muchas de las colonias que tras la aplicación de los tratamientos se mostraron libres de estos microsporidios, volvieron a mostrar infección pocos meses después. Por tanto, para el control a largo plazo de las nosemosis sería necesario realizar una serie de tratamientos periódicos durante la temporada apícola. Según resultados obtenidos sobre los niveles de infección, y apoyándonos en los datos de vitalidad y producción de las colonias, se puede sugerir que un tratamiento en otoño y otro en primavera podrían ser suficientes para controlar la infección por *Nosema* spp. a largo plazo en las colonias, y de este modo paliar los efectos negativos de la infección (bajada en población, cría, producción y en la tasa de supervivencia de las colonias). Además, nuestros ensayos de campo mostraron la necesidad de un consumo completo de los tratamientos aplicados en las colonias para conseguir una eficacia óptima de los mismos, ya que se observó una correlación positiva entre el consumo de fumagilina y el descenso en la carga parasitaria de *Nosema* en las colonias tratadas, al igual que ocurrió en estudios anteriores (Higes y col., 2011). Sin embargo, en un estudio reciente se afirma que los

tratamientos para controlar la infección por este microsporidio en las colonias no serían necesarios, basándose en una ausencia de efectos negativos de la infección por *N. ceranae* sobre la vitalidad (número de cuadros con cría) y productividad en las colonias de abejas infectadas (Traver y col., 2012). Sin embargo, en este estudio, la cantidad de miel producida no fue medida y no se realizó una comparación entre colonias infectadas y no infectadas, por lo que las conclusiones extraídas de estos resultados deberían ser interpretadas con cautela. En este sentido, otros autores tampoco encontraron una correlación entre la infección por *N. ceranae* y la variación en la vitalidad de las colonias (Genersch y col., 2010; Gisder y col., 2010; Stevanovic y col., 2011; Fernández y col., 2012), al contrario que lo mostrado por nuestros datos. Estos resultados contradictorios podrían reflejar diferencias en los procedimientos experimentales, ya que se utilizaron diferentes metodologías para medir el grado de infección, así como para evaluar la vitalidad y productividad de las colonias, por lo que sería necesario el establecimiento de una estandarización de procedimientos para el análisis y seguimiento de la infección por *Nosema* spp. en las colonias de abejas, con el fin de comparar de manera objetiva los resultados obtenidos en distintas regiones del mundo, y así entender mejor las diferencias epidemiológicas y patológicas que podría presentar estos microsporidios a lo largo del mundo.

Por otra parte, con respecto a la fumagilina, distintos estudios han mostrado una acción genotóxica y mutagénica de esta molécula en condiciones experimentales (Stanimirovic y col., 2007; COM, 2011), por lo que su impacto negativo sobre el estado sanitario de las abejas no es descartable. Además, estudios recientes han sugerido posibles efectos dañinos a corto plazo sobre el estado general de las colonias de abejas tras el tratamiento con este producto (Eischen y col., 2012). Por otro lado, la posibilidad de un efecto negativo de este antibiótico sobre la flora bacteriana de las abejas, y por tanto, sobre sus defensas contra determinados patógenos también debería ser evaluada, teniendo en cuenta que la microbiota intestinal es capaz de formar una barrera protectora esencial contra los patógenos intestinales (Koch y Schmid-Hempel, 2011). En cuanto a la seguridad alimentaria para los humanos, los estudios sobre los límites máximos de residuos en miel de la fumagilina serían esenciales para determinar la idoneidad de esta molécula como medicamento

veterinario para el tratamiento de las nosemosis en las colonias de abejas, así como para determinar la posología óptima en relación a la seguridad alimentaria.

Debido a que en la actualidad no existen tratamientos autorizados para el control de las nosemosis en muchos países del mundo, los estudios dirigidos a la búsqueda de nuevos tratamientos o mecanismos zootécnicos potencialmente activos contra las nosemosis son de vital importancia, con el fin de aportar soluciones al sector apícola para paliar los efectos dañinos de estas enfermedades en las colonias de abejas. Con este objetivo, en el Trabajo Experimental 2 se presenta un estudio preliminar que evalúa la eficacia de tres productos (Nosestat®, Salicilato de Fenilo y Vitafeed Gold®) frente a las nosemosis causadas por *N. ceranae* y/o *N. apis* en condiciones de campo. El análisis de la presencia de microsporidios en las colonias mostró a *N. ceranae* como la especie dominante, mientras que *N. apis* sólo apareció en 3 de las 40 colonias del ensayo tras la aplicación de los tratamientos, por lo que los resultados obtenidos deberían ser referidos principalmente a *N. ceranae*. Tras la aplicación de los productos ensayados se observó una ausencia de eficacia o eficacia muy limitada de los mismos en nuestras condiciones experimentales, ya que todos los grupos, incluido el grupo que recibió fumagilina, continuaron mostrando infección por *Nosema* spp. tras la aplicación de los mismos. Sin embargo hay que destacar que en todos los grupos que recibieron los tratamientos, la ausencia de un consumo apropiado de las distintas dosis suministradas a las colonias, sobre todo en la aplicación de otoño, pudo influir de manera notable en el desarrollo del ensayo y de los resultados obtenidos. En el caso de la fumagilina, estudios previos han demostrado la existencia de una correlación negativa entre el consumo de este producto y la proporción de abejas infectadas en las colonias (Higes y col., 2011; Botías y col., 2012c), lo cual podría ocurrir también con el resto de agentes terapéuticos. Este hecho evidencia la gran importancia de un consumo adecuado de los tratamientos suministrados, y por ello, del conocimiento de los factores que puedan interferir en ello, como por ejemplo las condiciones ambientales del momento de aplicación del producto, la presentación del producto de manera atractiva para las abejas o la solubilidad del producto en el excipiente seleccionado. De este modo, los estudios sobre la posología adecuada de aquellos agentes potencialmente terapéuticos para

controlar esta enfermedad son de vital importancia, y este ensayo supone un estudio preliminar que debería ser ampliado en el futuro. Asimismo, la adecuación del método de preparación y aplicación de los tratamientos a las condiciones reales de trabajo de los profesionales de la apicultura es un factor clave a tener en cuenta en la selección de una posología óptima.

Igualmente, el método utilizado para evaluar la eficacia de dichos productos fue el análisis de un grupo de abejas pecoreadoras (n≥30) antes y después de la aplicación de los mismos, observando la presencia de *Nosema* spp. en las muestras recogidas en ambos momentos. Dado que estudios previos han señalado el análisis de la proporción de abejas infectadas dentro de las colonias como un método más fiable para conocer la carga parasitaria por *Nosema* spp., y por tanto, para evaluar la eficacia de una sustancia activa (Higes y col., 2008a; Oliver, 2011; Botías y col., 2012a), la capacidad terapéutica real de estos productos se debería examinar con esta técnica.

La ausencia de diferencias significativas en la población de abejas adultas y la cantidad de cría entre los grupos a los que se administraron estas tres sustancias (Nosestat®, Salicilato de Fenilo, Vitafeed Gold®) y el grupo control (jarabe), puede indicar también una carga parasitaria equivalente en estos grupos, tal y como se describe en ensayos previos (Higes y col., 2008a; Soroker y col., 2011; Botías y col., 2012a). Únicamente las colonias tratadas con fumagilina mostraron una población significativamente mayor a la presentada por las colonias control en el momento de máxima actividad de las colonias (junio del 2009), lo cual podría ser un reflejo de la mayor eficacia relativa que mostró esta sustancia tras los tratamientos recibidos (comparado con el resto de los grupos). Aunque la eficacia de la fumagilina en el control de *N. ceranae* y *N. apis* ha sido probada en estudios previos (Gregorc y Sulimanovic, 1996; Higes y col., 2008a, 2011; Williams y col., 2008), en este ensayo únicamente el 30% de las colonias tratadas con esta sustancia tras el tratamiento de primavera 2009 mostraron estar libres de infección por *Nosema* spp.

La producción de miel fue significativamente mayor en el caso de las colonias tratadas con fumagilina en relación a aquellas a las que se suministró el producto Vitafeed Gold® y las colonias control. Este dato también podría ser un reflejo de la

mayor capacidad para controlar la nosemosis por parte de este antibiótico en nuestras condiciones experimentales, y por tanto, de mitigar los efectos subclínicos que provoca esta enfermedad. El no haber podido evaluar el nivel de consumo del producto Vitafeed Gold®, no permitió analizar de manera objetiva su capacidad como agente terapéutico para tratar las nosemosis.

Hasta el momento, son numerosas las sustancias que se han evaluado como alternativas al uso de la fumagilina para el control de las nosemosis (Békési y col., 2009; Maistrello y col., 2009; Nanetti, 2009; Tlak Gajger y col., 2009; Costa y col., 2010; Paldi y col., 2010; Porrini y col., 2010), y aunque varios de estos agentes terapéuticos han mostrado actividad frente a *Nosema* de manera significativa, éstos requieren de una confirmación de eficacia en condiciones de campo y de un estudio sobre los excipientes más apropiados con los que aplicar los mismos, lo cual debería abordarse en futuros estudios.

Para finalizar, la nosemosis tipo A parece encontrarse en una situación endémica en nuestro país y en otras regiones del mundo (Martín-Hernández y col., 2007, 2012; Giersch y col., 2009; Genersch y col., 2010; Yoshiyama y Kimura, 2010) y en las condiciones favorecedoras para su desarrollo y proliferación, esta enfermedad ha mostrado ser muy dañina para las colonias de abejas (Kauffeld, 1972; Fries, 1988; Yücel y Dorgaglu, 2001). Sin embargo, los estudios que han analizado la prevalencia de microsporidios en las colonias de abejas en los últimos años han encontrado una alta tasa de detección de *N. ceranae* en general (Chauzat y col., 2007; Klee y col., 2007; Martín-Hernández y col., 2007; Tapazsi y col., 2009; Stevanovic y col., 2010; Tlak Gajger y col., 2010) con respecto a la encontrada para *N. apis*. De este modo, *N. ceranae* parece haber incrementado de manera notable su prevalencia en un breve periodo de tiempo, y tanto en España como en otras áreas del mundo, este microsporidio respondería a una situación epidémica (Chen y col., 2008; Higes y col., 2010; Tlak Gajger y col., 2010; Stevanovic y col., 2011; Traver y Fell, 2011). Debido al descubrimiento relativamente reciente de *N. ceranae*, el impacto de este patógeno en las colonias de abejas melíferas se ha ido conociendo de manera paulatina y por ello, los factores desconocidos sobre la nosemosis tipo C son numerosos. Los estudios futuros probablemente aportarán la información necesaria para logar el control de

esta enfermedad emergente de las abejas melíferas. A su vez, los métodos de control de la nosemosis tipo C permitirán controlar y prevenir un posible incremento en la prevalencia y virulencia de *N. apis* en las colonias de abejas, ya que el efecto de los métodos de control y profilaxis para ambas enfermedades parece ser similar.

El control y prevención de las nosemosis en las colonias de abejas, apoyado en un conocimiento de los patrones epidemiológicos de estas enfermedades en las diversas condiciones, supondrá un gran beneficio para estos valiosos insectos sociales y por extensión, tanto para la humanidad como para la biodiversidad de nuestro planeta.

# CONCLUSIONES

**PRIMERA:** *N. ceranae* y *N. apis* presentan un patrón epidemiológico diferente, ya que el primero de ellos aparece en las colonias de abejas durante todo el año y presenta una mayor prevalencia tanto dentro de una colonia, como dentro de un apiario en nuestras condiciones experimentales, mientras que *N. apis* aparece preferentemente en los meses otoñales y primaverales del año y su prevalencia es menor en todas las escalas estudiadas.

**SEGUNDA:** La entrada de *N. ceranae* en las colonias de abejas melíferas en nuestro país probablemente se ha producido durante la última década, dándose un aumento significativo de su prevalencia en las poblaciones de abejas ibéricas en los últimos años. El hospedador original de este microsporidio es probablemente de origen asiático, y debido a la introducción y establecimiento de especies alóctonas procedentes de latitudes orientales en territorios con poblaciones de abejas melíferas libres de esta infección, *N. ceranae* ha sido capaz de saltar la barrera de un hospedador a otro. De este modo, este microsporidio es capaz de invadir un amplio rango de hospedadores del género *Apis*, entre los que se encuentra *A. koschevnikovi*, e incluso otros himenópteros del género *Bombus* autóctonos de Argentina. Además, dentro de la especie *A. mellifera*, este microsporidio tiene una amplia distribución, apareciendo no sólo en nuestro país, sino también en poblaciones autóctonas de *A. mellifera intermissa* del Norte de África (Argelia), y en las poblaciones introducidas de *A. mellifera* establecidas en países asiáticos como Indonesia y Corea del Sur, o en las Islas Salomón (Oceanía).

**TERCERA:** *N. ceranae* presenta diferencias en el potencial biótico con respecto a *N. apis*, admitiendo el primero un mayor rango de temperaturas en el cual es capaz de reproducirse y completar su ciclo biológico. Así, en las fases iniciales de la infección, a 33°C, el potencial biótico de ambas especies es similar, aunque en el caso de *N. ceranae* hay un mayor número de fases inmaduras (70%) que de esporas maduras, mientras que en *N. apis* a la misma temperatura, esta proporción entre fases es igual (50%). A 25°C y a 37°C el potencial biótico de *N. ceranae* es mayor que el de *N. apis*.

Además, una vez producida la infección en las células epiteliales del ventrículo, *N. ceranae* es capaz de continuar con su proliferación y desarrollo al exponer a las abejas infectadas a la temperatura de 37°C, mientras que *N. apis* ve inhibida su multiplicación a dicha temperatura.

**CUARTA:** Las egagrópilas de abejaruco (*Merops apiaster*) pueden actuar como fómites de esporas infectivas de *N. ceranae* para las abejas melíferas. Las esporas contenidas en estos fómites permanecen viables e infectivas tras 18 días de exposición de las egagrópilas a las condiciones climáticas del medio en que éstas fueron depositadas por los abejarucos.

**QUINTA:** El reemplazo inducido de la abeja reina en las colonias infectadas por *Nosema* spp. es capaz de reducir la carga parasitaria por estos microsporidios en las mismas, presentándose como una alternativa al uso de antibióticos para el control de las nosemosis en las colonias de abejas melíferas.

**SEXTA:** Las nosemosis pueden ser controladas mediante la aplicación de 120 mg. de fumagilina en primavera y en otoño, apreciándose la existencia de una correlación negativa entre el consumo de este medicamento y la carga parasitaria por *Nosema* spp. Sin embargo, este medicamento no es eficaz en el tratamiento a largo plazo de las nosemosis, ya que sólo 5 semanas tras la aplicación del mismo las colonias pueden resultar re-infectadas por estos microsporidios. Además, las colonias de abejas infectadas por *Nosema* spp. presentan un cuadro clínico caracterizado por un descenso significativo en la densidad de población, la cantidad de cría presente en las colonias y la cantidad de miel producida, así como un aumento en la proporción de celdillas de cría con respecto a la cantidad de abejas adultas.

# RESUMEN

Entre los factores que condicionan la rentabilidad de las explotaciones apícolas profesionales se encuentra la acción patógena de los microsporidios *N. apis* y *N. ceranae*, ya que se ha demostrado que causan pérdidas tanto en la viabilidad como en la productividad de las colonias infectadas.

En el presente trabajo comparamos los patrones epidemiológicos de ambos microsporidios. En una primera aproximación, estudiamos la dinámica de la infección en colmenares experimentales en los que se había detectado la presencia de ambas especies de *Nosema*. Así observamos que *N. ceranae* puede infectar abejas a lo largo de todo el año y presenta una mayor prevalencia tanto en el análisis de abejas individuales como en el número de colmenas infectadas en el apiario. Por el contrario, *N. apis* aparece preferentemente en los meses otoñales y primaverales y su prevalencia es siempre menor, manteniendo el patrón epidemiológico tradicionalmente descrito para este agente. También los datos obtenidos a través del análisis de mieles antiguas (del periodo 1988-2009) nos confirman que la introducción de *N. ceranae* fue reciente y gradual, ya que se detectó por primera vez en mieles del año 2000, lo que corrobora, junto al análisis de los datos de muestras clínicas de abejas, que el incremento en la detección de nosemosis en nuestro país observado en la última década se debe principalmente a la colonización de *A. mellifera* por parte de esta especie, *N. ceranae*.

Una vez denunciada en 1996 la infección de *Apis cerana* por *N. ceranae* en China, la distribución mundial observada *a posteriori* planteaba ciertas dudas sobre cuál podría ser su origen. En este sentido, la detección de *N. ceranae* en la especie asiática *A. koschevnikovi* y en poblaciones de *A. cerana* y *A. mellifera* en áreas geográficas en las que hasta el momento no se había encontrado este patógeno nos proporcionó evidencias de la amplia distribución de este microsporidio en dichas poblaciones, detectando además algunos signos que indican la condición de hospedador reciente de *A. mellifera* fuera de Asia. Por otro lado, el que *N. ceranae* se detecte en diversas especies del género *Apis* aporta indicios sobre su amplio rango de hospedadores y su extensa dispersión. Este hecho se confirmó posteriormente tras la detección de este microsporidio en himenópteros del género *Bombus* nativos de Argentina y en *A. mellifera intermissa* procedentes de colonias colapsadas de Argelia.

Las diferencias en los patrones epidemiológicos mostrados por *N. apis* y *N. ceranae* podrían estar relacionadas con el mayor potencial biótico de *N. ceranae*, ya que nuestros estudios demuestran que ésta última es capaz de completar su ciclo biológico a lo largo de un rango de temperaturas más amplio que *N. apis.* En cuanto a los factores epidemiológicos que

pueden contribuir a la gran dispersión de estos agentes, hemos observado que las egagrópilas de los abejarucos (*Merops apiaster* L., Meropidae) pueden actuar como fómites de *N. ceranae* ya que contienen esporas infectivas de este agente, lo que podría explicar la dinámica de propagación de las nosemosis dada la etología del ave.

Dadas las repercusiones negativas de las nosemosis tanto para la abeja individual como para las colonias, nos planteamos el estudio de diferentes métodos de control. Inicialmente y tras observaciones empíricas, evaluamos el reemplazo inducido de la abeja reina como método zootécnico para el control de estos agentes, comprobando que este reemplazo de la abeja reina (vieja) por una recién emergida y con mayor capacidad de puesta permite reducir la carga parasitaria de *Nosema* en las colonias infectadas en un nivel similar al conseguido tras la aplicación de fumagilina, por lo que éste se presenta como un método alternativo a la aplicación de sustancias activas. Los estudios con la fumagilina (única molécula registrada en ese momento en nuestro país) permitieron determinar la pauta de tratamiento más adecuada. Se comprobó que la aplicación de dos tratamientos al año (en otoño y primavera) con esta molécula puede controlar la parasitación, evitando los efectos perjudiciales sobre la vitalidad y productividad en las colonias de abejas. Tras la prohibición del uso de la fumagilina en julio del 2008, se hizo necesaria la búsqueda de tratamientos alternativos para el control de las nosemosis. De este modo evaluamos el efecto de tres posibles agentes terapéuticos Nosestat®, Salicilato de Fenilo y Vitafeed Gold® que, sin embargo, a las dosis ensayadas fueron ineficaces para el control de las nosemosis en las colonias infectadas.

El control y prevención de las nosemosis en las colonias de abejas, apoyado en un conocimiento de los patrones epidemiológicos de estas enfermedades en las diversas condiciones existentes supondrá un gran beneficio para la rentabilidad de las explotaciones apícolas profesionales y por extensión, la mayor supervivencia de estos insectos será muy positiva tanto para la humanidad como para la biodiversidad de nuestro planeta.

# SUMMARY

Some of the factors that can determine the profitability of professional beekeeping exploitations are the microsporidian pathogens *N. apis* and *N. ceranae*, since they cause losses to viability and productivity in the infected colonies.

In the present work, the epidemiological patterns of both microsporidia were compared. In a first approach, we studied the infection dynamics in experimental apiaries in which the presence of both *Nosema* species had been detected. Thus, we observed that *N. ceranae* can infect honey bees all year long, showing a higher prevalence both in individual bees and in the infected colonies from the apiary. On the other hand, *N. apis* is more present in the autumn and spring months and shows a lower prevalence, displaying an epidemiological pattern similar to what is traditionally described for this agent. Moreover, the obtained data from the analysis of old honeys (from 1988 to 2009) confirm that the introduction of *N. ceranae* in the Spanish apiaries has been recent and gradual, given its older detection in a honey sample from the year 2000. This fact, with the results from clinical honey bee samples, corroborates that the increase in the detection of nosemosis in the Spanish apiaries in the last decade is mainly due to the colonization by *N. ceranae*.

Since the first report of *N. ceranae* infection in *Apis cerana* from China in 1996, the later observed worldwide distribution of this microsporidium raised some questions about its origin. In this sense, the detection of *N. ceranae* in the Asian species *Apis koschevnikovi* and in *A. cerana* and *A. mellifera* populations in areas where this pathogen had not been previously identified provided evidences of its widespread occurrence in the Asian bee populations. Moreover, we detected signs of continued spread of *N. ceranae* in *A. mellifera* populations out of Asia, providing further support for the hypothesis of a recent invasion of the European honey bee by this microsporidium. Furthermore, the detection of *N. ceranae* in several *Apis* species indicates its widespread and extensive range of hosts, also confirmed through the detection of this microsporidium in three Argentinian native bumblebees and in collapsed colonies of *A. mellifera intermissa* from Argelia.

The differences in the epidemiological patterns shown by *N. apis* and *N. ceranae* may be related with the higher biotic potential of the latter, since it is able to complete its biological cycle through a wider range of temperatures than *N. apis*. Concerning the epidemiological factors related to their large dispersion, we observed that the regurgitated pellets of the bee-eaters (*Merops apiaster* L., Meropidae) contain infective spores of *N. ceranae* and thus can act as fomites of this pathogen, probably influencing the propagation dynamics of nosemosis given the ethology of this bird.

Due to the negative impact of nosemosis both at the individual and the colony level, we considered the goal of studying different control methods. Firstly, and after empirical observations, we evaluated the induced queen replacement as a zootechnical method for the control of these agents, proving that replacement of the old queen by a newly emerged one, with a greater egg-laying potential, reduces the parasitic load of *Nosema* in the infected colonies in a level similar to the achieved by the treatment with fumagillin. Therefore, this method is presented as an alternative to the application of active substances in the infected colonies. On the other hand, the studies with fumagillin (the only molecule registered at that moment in our country) indicated the most suitable guidelines for treatment. Indeed, the application of two treatments in a year (in autumn and in spring) with this molecule may control *Nosema* parasitization, avoiding the detrimental effects over the vitality and productivity of honey bee colonies. Finally, after the prohibition against the use of fumagillin in July 2008, the search of alternative treatments to control nosemosis was needed. Hence, we evaluated the effect of three possible therapeutic agents Nosestat™, Phenyl Salicylate and Vitafeed Gold™ which however, resulted ineffective to control nosemosis in the infected colonies at the tested dosages.

The control and prevention of nosemosis in the honey bee colonies, based on the knowledge of their epidemiological patterns in different field conditions will be beneficial for the profitability of professional beekeeping exploitations, and thus, for the mankind and the biodiversity of the planet.

# RÉSUMÉ

Parmi les facteurs qui influent sur la rentabilité des exploitations apicoles on trouve l´action pathogène des microsporidies *N. apis* et *N. ceranae*, puisqu´ils causent des pertes tant à la viabilité qu'à la productivité des colonies infectés.

Dans cette étude nous avons comparé le profil épidémiologique de deux microsporidies. Dans une première approche, nous avons étudié la dynamique de l´infection dans des ruchers expérimentaux où avait été détectée la présence des deux espèces de *Nosema.* Nous avons observé que *N. ceranae* peut infecter des abeilles tout au long de l´année. Sa prévalence est plus importante que celle de *N. apis* aussi bien quand on analyse des abeilles individuellement que quand on évalue le nombre de colonies infectées d'un rucher. Par contre, *N. apis* apparaît de préférence à l'automne et au printemps, et sa prévalence est toujours plus faible, tout en maintenant le profil épidémiologique traditionnellement décrit pour cet agent. Les résultats obtenus par l´analyse de miels anciens (1988-2009) nous confirment que l´introduction de *N. ceranae* a été récente et graduelle, car elle a été détectée pour la première fois dans un miel de l´année 2000. Ce résultat avec ceux qui proviennent des analyses des échantillons cliniques confirme que l´augmentation de la détection de la nosémose dans notre pays est surtout due à la colonisation d'*A. mellifera* par *N. ceranae*.

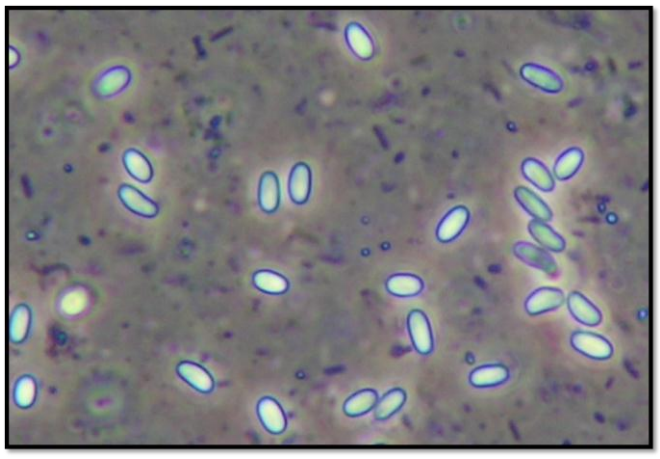
Une fois connue en 1996 l´infection de *N. ceranae* chez *Apis cerana* en Chine, sa distribution mondiale observée *a posteriori* posait certaines questions sur son origine. À cet égard, la détection de *N. ceranae* chez l´espèce asiatique *Apis koschevnikovi* et dans les populations de *A. cerana* et *A. mellifera* originaires de régions où ce pathogène n´avait pas été auparavant détecté, nous a apporté des preuves de la vaste distribution de cette microsporidie chez ces populations et de la condition d´hôte récent de *A. mellifera* en dehors de l´Asie. Par ailleurs, le dépistage de *N. ceranae* chez plusieurs espèces du genre *Apis* apporte des indices sur la variété des hôtes et la grande dispersion de cette microsporidie. Cela à été confirmé par la détection de *N. ceranae* chez des hyménoptères du genre *Bombus* natifs de l´Argentine et chez *A. mellifera intermissa* provenant de colonies effondrées de l´Algérie.

Les différences entre les profils épidémiologiques montrés par *N. apis* et *N. ceranae* pourraient être dues au plus grand potentiel biotique de *N. ceranae*, car nos résultats démontrent que cette microsporidie est capable de compléter son cycle biologique dans une plage de températures plus grande que *N. apis*. En ce qui concerne les facteurs épidémiologiques qui peuvent contribuer à la grande dispersion de ces agents, on a observé que les égagropiles des guêpiers d´Europe (*Merops apiaster* L., Meropidae) peuvent remplir la

fonction de fomites de *N. ceranae*, étant donné qu´ils contiennent des spores infectieuses de cet agent. Cela pourrait en partie expliquer la dynamique de propagation des nosémoses compte tenue de l´éthologie de l´oiseau.

Eut égard aux conséquences négatives des nosémoses pour l´abeille en tant qu'individu et pour les colonies, nous avons envisagé l'étude de différentes méthodes de contrôle. Tout d´abord, et après des observations empiriques, nous avons évalué le remplacement induit de la reine comme méthode zootechnique de contrôle. Nous avons vérifié que le remplacement induit de l'ancienne reine par une autre naissante ayant une capacité de ponte plus grande permet de réduire la charge parasitaire de *Nosema* à un niveau semblable à celui obtenu après l´application de fumagiline. Cette méthode représente une alternative à l´application de substances actives. Les études sur la fumagiline (la seule molécule autorisée à ce moment-là dans notre pays) ont permis de déterminer le protocole de traitement le plus approprié. Nous avons vérifié que l´application de deux traitements par an (à l´automne et au printemps) peut contrôler le parasitisme, et donc éviter les effets négatifs de *Nosema* sur la vitalité et la productivité des abeilles. Après l´interdiction de l´utilisation de la fumagiline en juillet 2008, il est devenu nécessaire de chercher des traitements alternatifs pour contrôler les nosémoses. Par conséquent, nous avons évalué l´effet de trois agents thérapeutiques Nosestat®, Phenyl Salycilate et Vitafeed Gold®. Aux doses essayées, tous trois ont cependant été inefficaces pour le contrôle des nosémoses chez les colonies infectées.

Le contrôle et la prévention des nosémoses chez les colonies d´abeilles, appuyés sur une connaissance des profils épidémiologiques de ces maladies dans les diverses conditions existantes sera bénéfique pour la rentabilité des exploitations apicoles professionnelles. La plus grande survie de ces insectes sera également très positive pour l´humanité et pour la biodiversité de notre planète.



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# **ANEXO:** Otros artículos

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ORIGINAL PAPER

# Comparison of the energetic stress associated with experimental *Nosema ceranae* and *Nosema apis* infection of honeybees (*Apis mellifera*)

**Raquel Martín-Hernández · Cristina Botías · Laura Barrios · Amparo Martínez-Salvador · Aránzazu Meana · Christopher Mayack · Mariano Higes**

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**Abstract** *Nosema ceranae* is a relatively new and widespread parasite of the western honeybee *Apis mellifera* that provokes a new form of nosemosis. In comparison to *Nosema apis*, which has been infecting the honeybee for much longer, *N. ceranae* seems to have co-evolved less with this host, causing a more virulent disease. Given that *N. apis* and *N. ceranae* are obligate intracellular microsporidian parasites, needing host energy to reproduce, energetic stress may be an important factor contributing to the increased virulence observed. Through feeding experiments on caged bees, we show that both mortality and sugar syrup consumption were higher in *N. ceranae*-infected bees than in *N. apis*-infected and control bees. The mortality and sugar syrup consumption are also higher in *N. apis*-infected bees than in controls, but are less than in *N. ceranae*-infected bees. With both microsporidia, mortality and sugar syrup consumption increased in function of the increasing spore counts administered for infection. The differences in energetic requirements between both *Nosema* spp. confirm that their metabolic patterns are not the same, which may depend critically on host–parasite interactions and, ultimately, on host pathology. The repercussions of this increased energetic stress may even explain the changes in host behavior due to starvation, lack of thermoregulatory capacity, or higher rates of trophallaxis, which might enhance transmission and bee death.

R. Martín-Hernández · C. Botías · M. Higes (✉)
Bee Pathology Laboratory, Centro Apícola Regional, JCCM,
San Martín s/n,
19180 Marchamalo, Guadalajara, Spain
e-mail: mhiges@jccm.es

L. Barrios
Statistics Department, CTI,
Consejo Superior Investigaciones Científicas,
28006 Madrid, Spain

A. Martínez-Salvador
Epidemiology Department, TRAGSEGA,
Madrid, Spain

A. Meana
Animal Health Department, Facultad de Veterinaria,
Universidad Complutense de Madrid,
28040 Madrid, Spain

C. Mayack
Department of Biology, Colorado State University,
Fort Collins, CO 80523, USA

## Introduction

It is obvious that, when a parasite outcompetes its host for the nutrient resources available, the host itself will suffer severe energetic stress. Infected hosts may appear to compensate for such a situation by feeding more, although they also are less efficient in obtaining energy than uninfected hosts. Therefore, not only do parasites take nutrients and energy away from hosts, but they also lower the rate at which energy becomes available for hosts to carry out vital functions (Walkey and Meakins 1970). Changes in feeding behavior following infection are just one example of the many potential alterations that might arise due to the need to assimilate more nutrients (Milinski 1985), and indeed, energetic stress typically underlies many of the physiological and behavioral alterations induced by infection (Milinski 1984).

Microsporidia are spore-forming fungal pathogens that develop as obligate intracellular organisms and infect a wide variety of hosts, ranging from insects to mammals (Adl et al. 2005). As they lack mitochondria and reproduce rapidly within a host cell, taking up ATP from their surroundings, parasitic microsporidia are particularly likely to exert severe

energetic stress on their hosts (Williams 2009). *Nosema apis* is a well-studied microsporidian gut parasite that has co-evolved with the western honeybee *Apis mellifera* and is known to cause a series of metabolic changes (Bailey 1981). However, the extent of the energetic stress induced on the honeybee through *N. apis* infection remains unclear. While a decrease in feeding has been observed following the infection of caged bees (Rinderer and Elliot 1977), in another study, food consumption increased with no increase in oxygen consumption, suggesting that the parasite itself is likely to be responsible for producing more hunger rather than the host augmenting its metabolic activity (Moffett and Lawson 1975). By contrast, *Nosema ceranae* is a relatively new microsporidian parasite of *A. mellifera* (Higes et al. 2006; Huang et al. 2007) that was found to cause energetic stress in honeybees, diminishing the survival of infected individuals (Mayack and Naug 2009). By comparison to *N. apis*, *N. ceranae* appears to be more virulent at both the individual and colony levels (Higes et al. 2008; Paxton et al. 2007). This increased virulence could reflect the fact that the parasite–host relationship of *N. ceranae* has evolved over a relatively short period and therefore it exerts more energetic stress on its host. The implications of energetic stress on virulence at the colony level must be taken into consideration since foragers carry a disproportionate parasite load following *N.* ceranae infection (Higes et al. 2008). About 30% of the colonies' energy is spent on foraging, and thus, a decrease in the energy available to individuals in association with the changes in metabolic rate for foraging following infection can strongly affect the energy balance at the colony level and, by consequence, the success of the colony (Harrison and Fewell 2002).

In recent reviews of *N. ceranae*, the need for more comparative studies of *N. apis* and *N. ceranae* was highlighted in order to distinguish the intrinsic mechanisms associated with the increased virulence observed in infected bees (Paxton 2010; Fries 2009). The virulence associated with each parasite–host complex can either drastically increase or decrease over time, and as a result, each must be studied in a case by case manner (May and Anderson 1990). Therefore, feeding experiments on caged bees have been performed to quantify the energetic stress induced by *N. apis* and *N. ceranae* infection. In addition, the mortality of the bees associated with either parasite has been monitored as an indicator of virulence.

## Methods

### *Nosema*-free honeybees for experimental infection

Frames of capped brood were obtained from a healthy colony of *Apis mellifera iberiensis* (*Nosema*-free confirmed by PCR) located in an experimental apiary 20 km from the "Centro Apícola", and they were kept in an incubator at 34°C (±1°C) to provide a supply of newly emerged *Nosema*-free honeybees. The emergent worker bees were carefully removed, and groups of 25 bees were confined to 40 different cages (175 mm long, 45 mm in diameter) that were kept in the incubator for 5 days. The bees were fed ad libitum with a sucrose solution (50% *w*/*w* in distilled water) combined with 2% Promotor L (Calier Lab.), a commercial mixture of amino acids and vitamins. Honey and pollen were not used to feed the bees to avoid possible contamination with infective *Nosema* spp. spores.

### Production of viable *N. apis* and *N. ceranae* spores

*N. apis* and *N. ceranae* spores were obtained from experimentally infected bees as described previously (Higes et al. 2007). Spores were isolated from adult honeybee samples of naturally infected Spanish honeybee colonies sent to our laboratory for pathological studies. The abdomens of bees were macerated in distilled water (PCR grade) using a sterile manual tissue grinder. The ground contents were filtered through a Whatman mesh (no. 4), and the resulting suspension was re-suspended in distilled water (PCR grade), which was then centrifuged. Each time a pellet of mature spores formed at the bottom of the tube, the liquid at the top was discarded. This procedure was repeated three times to remove all the contaminating debris, and the spores were then quantified using a hemocytometer (OIE 2008), while the *Nosema* species was confirmed by PCR (Martín-Hernández et al. 2007). The spores were divided into batches, stored in distilled water, and they were maintained at a constant temperature until they were used to induce infection.

### Experimental design to determine energy demands and survival

Experimental infection was induced in 5-day-old bees as described previously (Higes et al. 2007), and the experimental groups were classified as indicated in Table 1. The concentrations of *N. apis* or *N. ceranae* spores administered were 0, $10^3$, $10^4$, $5\times10^4$ and $10^5$ spores per bee, and each of the ten groups was established with four replicates of 25 bees in a cage.

Before placing the bees in the cages, the bees were starved for 2 h, and then, they were fed individually with 2 μl of 50% sucrose solution containing the appropriate concentration of the inoculum. To achieve the correct dosage, honeybees were anesthetized with $CO_2$ for ease of handling. When each bee woke up, the droplet of 50% sucrose solution mixed with the spores was administered by touching a micropipette to the bee's mouthparts until the

**Table 1** The various amounts of spore doses administered at the beginning of the experiment for *N. apis* and *N. ceranae* species of parasite

| Spore dose (spores/bee) | Group name | |
|---|---|---|
| | *N. apis* | *N. ceranae* |
| 0 | A1 | C1 |
| $10^3$ | A2 | C2 |
| $10^4$ | A3 | C3 |
| $5 \times 10^4$ | A4 | C4 |
| $10^5$ | A5 | C5 |

entire droplet was consumed. Bees that did not consume the entire droplet were discarded (Malone et al. 1999). Uninfected control bees were fed with 2 μl of the 50% sucrose solution alone.

After inducing infection, the bees in each cage were fed ad libitum with a sugar syrup made up of 50% sucrose solution with 2% Promotor L (Calier Lab.) through an individual feeder attached to the cage. In order to measure the nutritional demand of infected and uninfected bees, the amount consumed was used as a measure of energetic stress, as described previously (Mayack and Naug 2009). On days 1 (D1), 2 (D2), and 6 (D6) post-infection (p.i.), the amount of syrup consumed by each cage of bees was recorded by weighing the feeder, and the mean amount consumed per bee at each time point was then calculated (on the 6th day p.i., the total ingested food was calculated as the daily average consumed food). Two different incubators (Memmert® Mod. IPP500, ±0.1°C) were maintained at 33°C; one contained the *N. ceranae*-infected bees, and the other, *N. apis*-infected bees, in order to avoid cross-contamination. In addition, a group of uninfected bees were kept in each of the two incubators with the infected bees. The cages were observed daily for bee mortality, and all dead bees were removed when detected.

On the seventh day post-infection, all the remaining living bees were sacrificed by freezing, and the entire abdomen was removed to confirm the *Nosema* species present using the multiplex PCR method described previously (Martín-Hernández et al. 2007). Bees of both control groups served as negative controls and confirmed the absence of infection.

### Statistical analysis

A generalized linear model (GLM) was used to study the dependent variables (syrup consumption at three different time points, D1, D2, and D6) and the effects of two fixed factors. These factors included infection at three levels (no infection, infection with *N. apis*, infection with *N. ceranae*), the spore dose administered, and the model used (intercept, infection, and nested spore dose in infection). The differences were calculated to a 95% confidence level with a Wald chi-square test, and the same test was used to estimate the parameters selected to compare the effect between each dose and to calculate the differences between them.

The data were represented in bar graphs. The differences in syrup consumption on the 3 days post-infection were averaged according to the spore doses administered, and then, the differences between the spore doses administered averaged over the 3 days were compared using repeated measures ANOVA with a post hoc Bonferroni analysis.

## Results

*N. ceranae*-infected bees consumed significantly more syrup than *N. apis*-infected or uninfected bees throughout the experiment. In infected bees, the amount of syrup consumed increased with the dose of the *N. ceranae* spore inoculum at all the time points analyzed.

### Nutritional demands

The overall amount of syrup consumed increased significantly up to day 2 p.i., but it decreased significantly from days 2 to 6 p.i. On days 3, 4, and 5 p.i., the amount of syrup fed to the bees was not recorded as the quantity consumed was negligible. On day 1 p.i. (D1 time point), the fixed factors of the GLM showed an effect of infection on syrup consumption in the bees (Wald chi-square=480.2; $p<0.0001$), and on the 2nd day p.i. (D2 time point), the results were similar to those observed on D1. On the 6th day p.i. (D6 time point), only *N. ceranae*-infected bees consumed more syrup than *N. apis* and uninfected bees (Wald chi-square=45.1, $p<0.0001$; Fig. 1).

### Parasite load

As the spore dose increased, there was a significant increase in the amount of syrup consumed by infected bees (Wald chi-square=152.3; $p<0.0001$). At all spore doses, *N. ceranae*-infected bees consumed significantly more syrup than *N. apis*-infected or uninfected bees, and *N. apis*-infected bees consumed more syrup than uninfected ones. Considering the combined effects in *N. ceranae*-infected bees (*Nosema* infection and spore dose), the highest amount of syrup consumption was recorded in bees infected with the largest dose of spores, and a dose of $10^5$ spores per bee was the only dose that produced significantly higher consumption than the rest of the spore doses assayed (Wald chi-square=104.7, $p<0.0001$). The same was true for *N. apis* infection except that the highest amount of spores

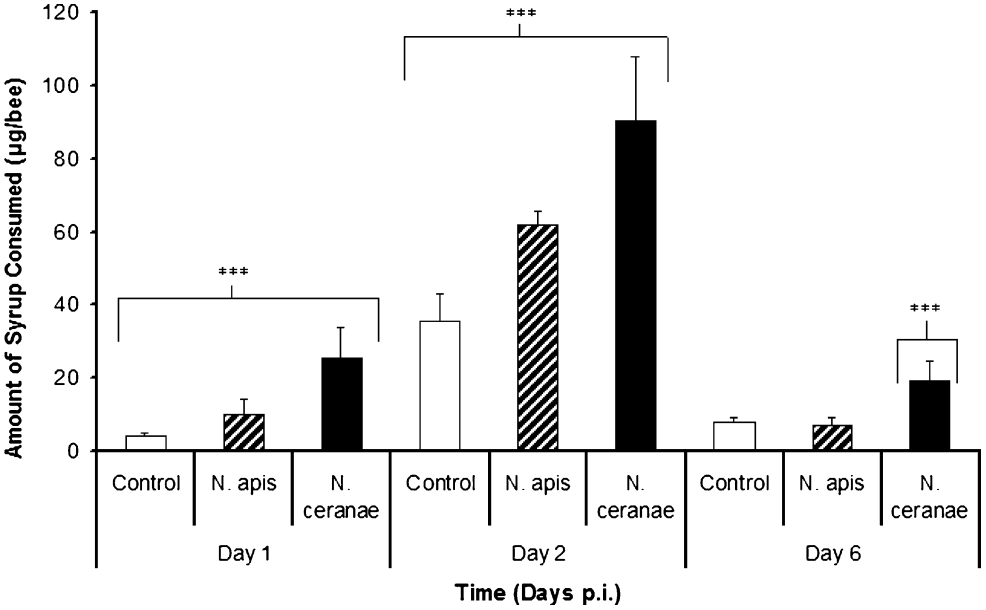


**Fig. 1** The amount of syrup consumed on the 3 days post-infection (days 1, 2, and 6 p.i.) by *N. apis* (*N*=16; 4 replicates×4 spore doses for each day; *striped bars*) and *N. ceranae* (*N*=16; 4 replicates×4 spore doses for each day; *solid bars*) compared to their respective control group (*N*=4; 4 replicates for day and *Nosema* species; *clear bars*). For clarity, the infected data are pooled across the 1,000–100,000 spore parasite loads administered at the beginning of the experiment. The data represent the mean values for each group, and their *standard deviation bars* and the *multiple asterisks* indicate highly significant differences between means tested at the 0.05 alpha level

administered was not significantly different from the rest (Wald chi-square=22.6, $p<0.0001$; Fig. 2).

## Bee mortality

Honeybee mortality was assessed at different time points during the experiment (Table 2), and no mortality was observed until day 6 p.i. in some of the infected groups. The mortality rates of infected bees showed a similar pattern to their overall energetic demands and the corresponding *Nosema* spore doses. Not only did bees infected with the higher doses of *N. apis* spores suffer higher mortality, but even greater mortality was seen in those infected with *N. ceranae* spores. As such, the highest mortality was evident in the bee group infected with the largest dose of *N. ceranae* spores ($10^5$ spores/bee), which produced 60.7% of death among the bees 6 days p.i. Indeed, mortality reached 93.1% after 7 days p.i. in this group of bees, at which time the mortality was higher in all *N. ceranae*-infected groups than in the control groups. In the case of *N. apis* infection, only the higher doses of spores ($10^5$ and $5\times10^4$ spores/bee) produced more mortality than in the control group of bees.

## Discussion

These results presented here clearly show that *N. ceranae* imposes greater energetic stress on infected bees, suggestive of a stronger virulence in comparison to *N. apis*.

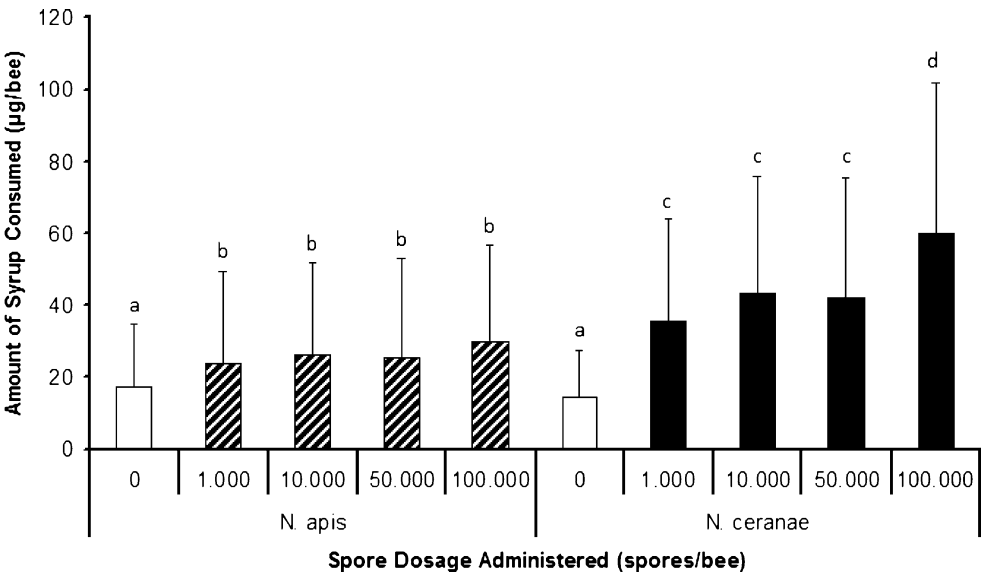


**Fig. 2** The amount of syrup consumed by bees infected with *N. apis* (*N*=12; 4 replicates×3 days for spore dose; *striped bars*), *N. ceranae* (*N*=12; 4 replicates×3 days for spore dose; *solid bars*), and their respective controls (*N*=12; 4 replicates×3 days; *clear bars*), plotted as a function of the 1,000–100,000 parasite load administered at the beginning of the experiment. For clarity, the infected data are pooled across the three measurements post-infection. The data represent the mean values for each group with the *standard deviation bars* and *letters* indicating significant differences between means at the 0.05 alpha level

**Table 2** Mortality (mean ± standard deviation) of infected and control groups

| | | | Days | | | |
|---|---|---|---|---|---|---|
| | | | 1 day | 2 days | 6 days | 7 days |
| Group | *Nosema* spp. | Spore dose | Mortality (%) | | | |
| A1 | Uninfected | 0 | 0 | 0 | 0 | 4.8±0.9 |
| A2 | *N. apis* | $10^3$ | 0 | 0 | 0 | 4.9±1.1 |
| A3 | *N. apis* | $10^4$ | 0 | 0 | 0 | 5.2±0.8 |
| A4 | *N. apis* | $5 \times 10^4$ | 0 | 0 | 10.6±2.7 | 21.6±1.7 |
| A5 | *N. apis* | $10^5$ | 0 | 0 | 17.9±2.1 | 31±0.9 |
| C1 | Uninfected | 0 | 0 | 0 | 0 | 4.1±1.1 |
| C2 | *N. ceranae* | $10^3$ | 0 | 0 | 0 | 11.1±2.1 |
| C3 | *N. ceranae* | $10^4$ | 0 | 0 | 8.4±1.8 | 20.1±0.9 |
| C4 | *N. ceranae* | $5 \times 10^4$ | 0 | 0 | 22.6±1.2 | 67.4±1.3 |
| C5 | *N. ceranae* | $10^5$ | 0 | 0 | 60.7±3.6 | 93.1±1.6 |

The mortality was calculated as the percentage of the caged bees dead at each time point

Energetic stress is related to the increasing doses of spores for infection, and it is highest in *N. ceranae*-infected bees, which also suffer the worst survival. Furthermore, these data are consistent with previous studies that demonstrate the detrimental effects of *N. ceranae* on individual bees and on colony survival as a whole (Higes et al. 2008; Mayack and Naug 2009; Naug and Gibbs 2009; Alaux et al. 2010). Energetic stress imposed by *N. ceranae* is much more substantial than previously thought, and it persists for much longer, lasting for up to 6 days post-infection.

The severe energetic stress observed in *Nosema*-infected bees is probably due to the parasite itself competing directly with its host for key nutrients and energy resources, like most microsporidian species. *N. ceranae* and *N. apis* only develop when in direct contact with the host cell cytoplasm (Fries et al. 1996; De Graaf et al. 1994; Weidner et al. 1999; Higes et al. 2007; Chen et al. 2009a), indicating that the parasite may require some external energy supply to reproduce (Weidner et al. 1999). Microsporidia lack mitochondria, and they have long been suspected to either take up ATP from the host cell environment or gain ATP by metabolizing host cell carbohydrates through the glycolytic pathway (Weidner et al. 1999). Consistent with this notion of ATP uptake, microsporidia have frequently been seen to be surrounded by host mitochondria, which probably facilitate the uptake of ATP from host cells (Dufort et al. 1987; Sokolova et al. 1988; Williams 2009).

In these experiments, the parasite dependence on host energy is manifested by the increase in syrup consumption by infected bees, as seen in earlier studies (Mayack and Naug 2009; Alaux et al. 2010). However, it is interesting to note that we found significantly higher feeding in *N. ceranae*-infected bees than in *N. apis*-infected bees. Some years ago, it was found that heavily infected cells of the gut lining may either be dead or dying, provoking poor nutrient absorption in the midgut of the bee and eventually leading to the early death of bees due to starvation (Liu 1984). Intriguingly, there was no difference in virulence between *N. apis*- and *N. ceranae*-infected bees fed ad libitum when administered the same doses of spores as in this experiment (Forsgren and Fries 2010) probably due to differences in the method of infection in the laboratory (e.g., house adult worker bees obtained from combs versus newborn bees obtained in an incubator, etc.). Therefore, the stronger virulence (mortality) of *N. ceranae*-infected bees at the same parasite load as *N. apis* is probably due to the severe energetic stress caused by the increased lack of nutrient absorption, which is likely to be due to more aggressive destruction of the gut lining. As a matter of fact, the degeneration of epithelial ventricular cells of the gut lining is more severe in *N. ceranae*-infected bees than in *N. apis*-infected bees, diminishing nutrient absorption (Higes et al. 2007) The more aggressive damage to the gut lining in *N. ceranae*-infected caged worker bees has also been detected in naturally infected worker and queen bees (Higes et al. 2008; 2009a, b).

Malnutrition that results in a higher within-host parasite load does not necessarily suppress immunocompetence that leads to a higher number of infected individuals in the population, as seen with microsporidia infecting vertebrate hosts (reviewed by Wakelin 1989 and Lloyd 1995). Invertebrate generation times are much shorter than those of vertebrates, and infection of invertebrates is usually chronic. Invertebrate parasites depend strongly on host resources (Pulkkinen and Ebert 2004), and thus, in some instances, severe starvation limits the growth of the parasite within invertebrate hosts. Indeed, severe energetic stress was shown to impede parasite spread and the decrease of the number of infected individuals at a population level when *Daphnia* was infected with the microsporidian *Glugoides intestinales* (Pulkkinen and Ebert 2004). However, this does not appear to be the case in this study, where consuming significantly more syrup over the period tested probably yields enough

additional energy to support the reproduction and larger parasite load of *N. ceranae*-infected bees (Chen et al. 2009b).

The severe energetic stress caused by *N. ceranae* when compared with *N. apis* may be related to the immune suppression seen in infected bees. Indeed, there is a clear trade-off between energy acquisition and activation of the insect's immune system to fight infections (Schmid-Hempel 2005). Short-term food deprivation in insects leads to a downregulation of the immune system, resulting in less resistance when challenged with infection. When fed under ideal conditions, the immune system is restored, demonstrating the energetic cost associated with maintaining an effective immune system (Siva-Jothy and Thompson 2002; Feder et al. 1997). In contrast to the activation of the immune system by *N. apis*, *N. ceranae* causes immune suppression (Antúnez et al. 2009), much like the *Varroa* mite that induces energetic stress and facilitates multiple co-infections in honeybees (Gregory et al. 2005; Yang and Cox-Foster 2005).

It is important to note that energetic stress was observed in infected bees maintained under ideal laboratory conditions, while under natural conditions, a combination of other factors may represent additional negative influences on honeybees. For example, at sub-lethal levels, pesticides like neonicotinoids have been shown to cause a further increase in energetic stress in *N. ceranae*-infected honeybees (Alaux et al. 2010). Energetic stress has important implications for the success of bee foraging since carbohydrates are their main source of fuel for flight, and foraging is a metabolically expensive activity (Rothe and Nachtigall 1989). Moreover, energetic stress is suspected to be the cause of the poor thermoregulation in infected foragers when they are chilled, and infected bees seek warmer locations within the hive to compensate when they feel cold. This deficient thermoregulation increases the probability of foragers suffering hypothermia, leading to their incapacity to sustain flight and provoking forager starvation outside of the colony (Campbell et al. 2010). Indeed, free-flying foragers infected with *N. ceranae* have lower hemolymph trehalose levels due to energetic stress, and based on the differences in sugar levels, it would be predicted that infected foragers could only fly two thirds the distance of an uninfected forager (Mayack and Naug 2010). The fact that the pollinators' habitat is declining adds yet further stress on honeybee foragers, causing an increase in the distance a honeybee forager has to fly to collect nectar and pollen for the colony (Naug 2009).

Not only would *N. ceranae* infection have some individual behavioral effects on foragers, but the colony could also be affected. Increased hunger due to *N. ceranae* infection leads to differences in trophallaxis rates within the colony, which may increase the rate of transmission and the spread of the disease (Feigenbaum and Naug 2010; Naug and Gibbs 2009). An increase in an individual's hunger through infection may increase the overall rate of foraging in the colony, as well as the colony's energetic demand regulating foraging. Indeed, hunger at the level of individual foragers within the colony increases foraging rates (Howard and Tschinkel 1980; Toth et al. 2005). Moreover, it is probable that vitellogenin levels can be indirectly modulated by nutritional stress, thereby inducing infected bees to start foraging earlier (Amdam and Omholt 2003; Nelson et al. 2007). The probability of infected foragers starving to death would also increase as they might be unable to fly back to the hive due to energetic stress (Mayack and Naug 2009; Naug 2009). As such, starvation may contribute to colony depopulation, highly infected foragers having been found dead far from their hives (Higes et al. 2008).

## Conclusion

This study contributes to the growing body of literature demonstrating that *N. ceranae* is more virulent than *N. apis* (reviewed in Higes et al. 2010). The increased virulence associated with the energetic stress observed could be due to the shorter co-evolution of the *N. ceranae* parasite–host complex, given that this is a relatively new parasite of the western honeybee when compared to *N. apis* (Higes et al. 2006; Klee et al. 2007). As described here and elsewhere, *N. ceranae* has a relatively important influence in terms of the fitness of its host, both alone or in combination with other agents. *N. ceranae* is probably less efficient in terms of the physiological integration of the host–parasite complex, and it must draw more food from its host due to less efficient energy conversion. Therefore, it is plausible that the stronger virulence observed following *N. ceranae* infection is due to additional energetic stress imposed by this relatively new parasite, over and above that of *N. apis*. Individual energetic stress from infection may have more far-reaching effects in honeybees as they are social insects, affecting the regulation of foraging or immune function, which may potentially affect the survival of infected bees.

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# *Nosema* spp. parasitization decreases the effectiveness of acaricide strips (Apivar®) in treating varroosis of honey bee (*Apis mellifera iberiensis*) colonies

**Cristina Botías,[1] Raquel Martín-Hernández,[1,2] Laura Barrios,[3] Encarna Garrido-Bailón[1] Antonio Nanetti,[4] Aranzazu Meana[5] and Mariano Higes[1]***

[1]*Laboratorio de Patología Apícola, Centro Apícola Regional, CAR, Junta de Comunidades de Castilla La Mancha, 19180 Marchamalo, Spain.*

[2]*Instituto de Recursos Humanos para la Ciencia y Tecnología, INCRECYT, Parque Científico de Albacete, Spain.*

[3]*Departamento de Estadística, CTI, Consejo Superior Investigaciones Científicas, 28006 Madrid, Spain.*

[4]*Consiglio per la Ricerca e la sperimentazione in Agricoltura, Unità di ricerca di apicoltura e bachicoltura, CRA-API, Via di Saliceto 80, 40128 Bologna, Italy.*

[5]*Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.*

## Summary

**Given the key role played by honey bees in almost all terrestrial ecosystems, maintaining bee populations in adequate sanitary conditions is crucial for these essential pollinators to continue their work. From the beginning of the 21st century, beekeepers have reported a progressive increase in the overwintering mortality of honey bee colonies worldwide. Despite the failure to reach a consensus regarding the cause of this phenomenon, pathogens are thought to be strongly implicated. In the present work, we provide evidence of the negative effects of colony parasitization by *Nosema* spp. – primarily by *N. ceranae* – on the effectiveness of acaricide strips to treat *Varroa destructor*. The effectiveness of the *Varroa* mite strip treatment (Apivar®) was greater in colonies in which *Nosema* spp. parasitization had been controlled. Several studies report that infection by *Nosema* spp. may affect the behaviour of worker bees. As the effectiveness of *Varroa* strip treatment depends on bees contacting the strips and their subsequent interaction within the colony, such behavioural and social alterations could interfere with the treatment and allow more severe effects to develop in the colonies infected by *Nosema*. These results should be considered when assessing acaricide treatments in field conditions due to the high prevalence of both pathogens worldwide.**

Received 27 July, 2011; accepted 17 September, 2011. *For correspondence. E-mail mhiges@jccm.es; Tel. (+34) 949 250 026; Fax (+34) 949 250 176. None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper. Trademarks are mentioned for scientific purposes only and this work does not explicitly recommend any of these products.



## Introduction

Honey bees (*Apis mellifera* L.) are important pollinators in agricultural settings worldwide and exert a strong influence in many natural ecosystems (Bradbear, 2009). Thus, maintaining populations in adequate sanitary conditions is crucial to ensure that they continue to perform these essential pollination activities.

The worldwide beekeeping sector has been faced in recent years with a notable increase in honey bee mortality and, although the underlying causes remain unclear, multifactorial processes involving different pathogens and parasites are thought to be responsible (European Parliament, 2010; vanEngelsdorp and Meixner, 2010; Higes *et al*., 2010a).

One of the most prevalent pathogens implicated in honey bee colony losses is the microsporidium *Nosema ceranae* (Higes *et al*., 2006; 2008; 2009; Bacandritsos *et al*., 2010; Bromenshenk *et al*., 2010; Suwannapong *et al*., 2010; Hatjina *et al*., 2011), an aetiological agent of an emerging illness now known as type C nosemosis (Higes *et al*., 2010b). Another highly prevalent pathogen related with colony losses in Europe and the USA is the ectoparasitic mite *Varroa destructor* (Amdam *et al*., 2004; Le Conte *et al*., 2010; Rosenkranz *et al*., 2010). Both pathogens are associated with a shortened life span of worker bees, changes in their flight behaviour, disorientation and colony loss (Kovac and Crailsheim, 1988; Kralj and Fuchs, 2006; 2010; Rosenkranz *et al*., 2010; Higes *et al*., 2010b). Indeed, *V. destructor* can cause colony

collapse in a period of 1–3 years if not properly treated (Rosenkranz *et al*., 2010). Similar effects have been described in honey bee colonies infected by *N. ceranae* in temperate climates (Higes *et al*., 2008; Bacandritsos *et al*., 2010; Borneck *et al*., 2010). Thus, the combination of both pathogens and associated viruses could considerably increase the risk of collapse in infected colonies (Higes *et al*., 2010a).

The *Varroa* mite control can be carried out by a variety of different acaricide products, including synthetic acaricides, organic acids and essential oils, or through biotechnical and biological methods (reviewed by Rosenkranz *et al*., 2010). In temperate regions like the Mediterranean area (as Spain), where the honey bee brood is almost constantly present in the hives, mites can multiply and spread rapidly. Hence, products with long-term efficacy such as synthetic acaricide strips provide the best form of chemical varroosis control, being the main treatment used by Spanish beekeepers (Source: Veterindustria and Laboratory Companies). These treatments consist of rigid or semi-rigid plastic polymer strips impregnated with acaricide, and their efficacy is dependent on the rubbing of the bees with the strips and the subsequent contact and interaction with other bees to distribute the active ingredient throughout the colony.

In recent years in Spain and other countries, the effectiveness of acaricide strips to treat *Varroa* mite infestation has decreased, and in general, only a mite resistance to acaricides has been proposed as source of the problem (Elzen *et al*., 2000; Mozes-Koch *et al*., 2000; Martin, 2004; Pettis, 2004). Indeed, few other possible causes for this loss of effect have been suggested (Trouiller, 1998; Floris *et al*., 2001).

Given the high prevalence of both *N. ceranae* and *V. destructor* in honey bee colonies worldwide, interaction between both pathogens should be considered. Thus, in the present study we focus on the impact of the *Nosema* infection over the effectiveness of a contact-dependent form of *Varroa* mite treatment (Apivar®) in honey bee colonies.

## Results and discussion

This study provides evidence of the negative impact of parasitization by *Nosema* spp. on the effectiveness of an acaricide treatment to combat the *Varroa* mite in honey bee colonies. As *N. ceranae* was present throughout the experiment in all infected colonies, it would appear that this effect may be primarily mediated by this microsporidium.

In September 2007, all the 50 colonies tested positive for both *V. destructor* and *N. ceranae*, with 26 of them (52%) co-infected with *N. apis* (14 colonies of group FUM and 12 of group SYR; Fig. 1), indicating that natural parasitization had occurred over a three-month period. The ease and speed with which these pathogens showed to spread in the present assay may be indicative of the problem currently facing Spanish beekeepers on a national level (Higes *et al*., 2010a).

The application of the *Nosema* spp. treatment in the group FUM revealed a high level of effectiveness ($\chi^2 = 28.4$, $P < 0.001$; Figs S1 and S2), as reported elsewhere (Higes *et al*., 2011). Indeed, in 77% ($n = 23$) of the treated colonies *Nosema* spp. was not detected by PCR in forager bees collected after treatment. However, 20% ($n = 6$) of the remaining colonies were infected with *N. ceranae* alone and 3% ($n = 1$) remained co-infected with both *Nosema* species. As no colonies were infected with *N. apis* alone, we gained no information as to the relative effectiveness of the antibiotic in treating the two distinct *Nosema* spp.

Conversely, all of the colonies administered with the vehicle alone (sugar-syrup) remained infected all throughout the trial, being 80% ($n = 16$) of the colonies infected with *N. ceranae* and 20% ($n = 4$) with *N. ceranae* and *N. apis* after syrup application (Fig. 2).

The analysis of *Nosema* species revealed a higher prevalence of *N. ceranae* with respect to *N. apis*, with *N. ceranae* present in all *Nosema*-infected colonies, in agreement with previous findings from several countries (Klee *et al*., 2007; Chen *et al*., 2008; 2009; Higes *et al*., 2009; Tapaszti *et al*., 2009; vanEngelsdorp *et al*., 2009). Indeed, in November (after fumagillin treatment), all the remaining infected colonies ($n = 7$ and $n = 20$ in the groups FUM and SYR respectively) exhibited *N. ceranae* infection, while only 18.5% ($n = 5$) were co-infected with *N. apis*. However, in the present experiment, the observed decrease in the prevalence of *N. apis* from September (52%) to November (18.5%) coincided with a notable drop in the average monthly temperatures (from 18.31°C in September to 5.13°C in November), so this fact may have been due to the hardening in the weather conditions, as infection by this microsporidium tends to decrease during the winter (Bailey, 1955).

On the other hand, the mean daily *Varroa* mite mortality was recorded as an indicator of the *Varroa* population in the honey bee colonies. Prior to treatment with acaricide strips there were no significant differences in mean daily *Varroa* mite natural mortality between the groups (*t*-test, $F = 1.55$, $P = 0.202$; Fig. 3A). Once the acaricide strips treatment was applied, the mean daily mortality of *Varroa* mites through the first 6 weeks tended to be higher in the group FUM, and significant differences were observed between the two experimental groups during the third and fourth weeks of treatment (*t*-test, $F = 7.74$, $P = 0.011$; Fig. 3B), suggesting that the effectiveness of this acaricide may have diminished in colonies parasitized by *Nosema* spp. (mainly *N. ceranae*).

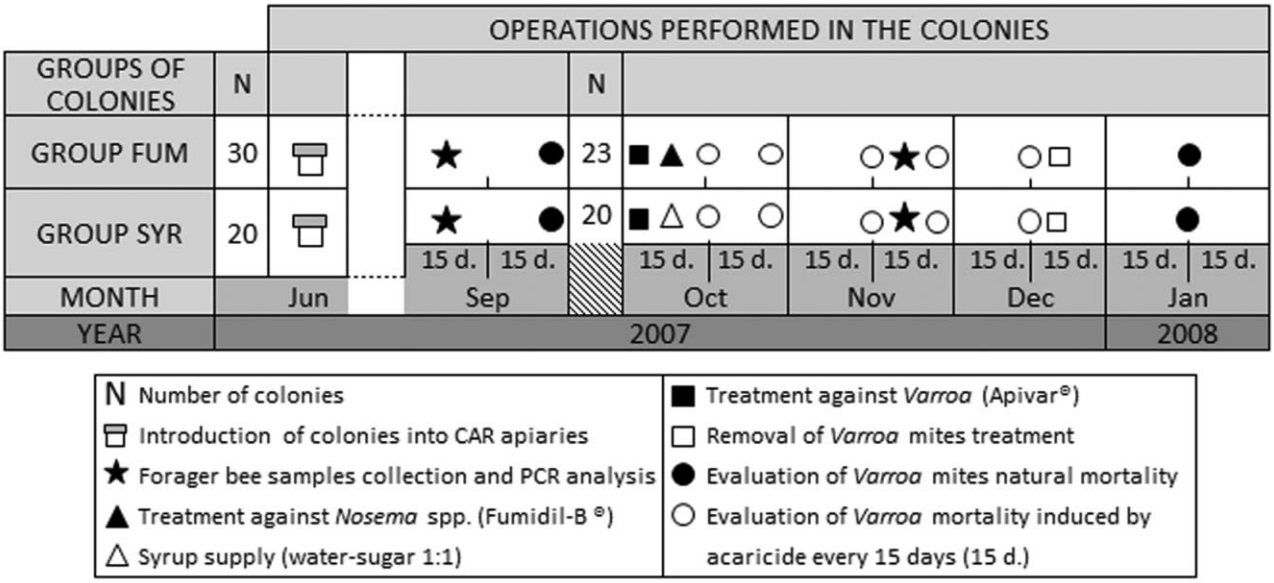


**Fig. 1.** Experimental procedures. Fifty homogenous colonies of *Apis mellifera iberiensis* (8–10 combs covered by adult bees and 3–4 frames of brood) were placed in one apiary of the 'Centro Apícola Regional' (CAR, Marchamalo, Spain) in June 2007 and all of them had queens born in the previous spring. Upon introduction of the colonies, evaluation of American Foolbrood, European Foolbrood and Chalkbrood disease was performed by confirming the absence of clinical symptoms and the absence of *Nosema* spp. and *V. destructor* was confirmed using standardized methods (OIE, 2004; Martín-Hernández *et al*., 2007). Colonies were located at a distance of 500 m away from another apiary naturally parasitized by both *Nosema spp.* and *V. destructor*, which acted as a natural parasites source for the new honey bee colonies. Upon confirmation of *Nosema* spp. and *V. destructor* parasitization in the 50 experimental colonies (September 2007), these were randomly transferred to two different apiaries: the group FUM in apiary 1; and the group SYR in apiary 2. These two apiaries, located 1 km apart, were also isolated from other professional apiaries to avoid interference due to the drifting behaviour of the bees (i.e. re-parasitization between groups or apiaries). On 9 October 2007, the following interventions were carried out: (i) Group FUM (treated): 30 colonies received four weekly treatments against nosemosis with Fumidil B® (Ceva Santé Animale), 1.5 g each dose, corresponding to 30 mg of fumagillin dissolved in 250 ml of sugar syrup (1:1), as described elsewhere (Higes *et al*., 2011); (ii) Group SYR (untreated controls): 20 colonies each received four weekly doses of 250 ml of the vehicle alone (sugar syrup). All interventions were administered in plastic bags placed over the brood chamber and syrup consumption was assessed weekly. On the day of administration of a new dose, the remaining unconsumed doses were removed from the colony and volume measured. Irrespective of the group, on 9 October all the colonies received also an Apivar® treatment to control the varroa infestation, as is described below (see Fig. 3 legend).
Meteorological data were got from the closest weather station CREA-SIAR (http://crea.uclm.es/~siar/datmeteo/resu_hist.php) all through the study.

Moreover, 1 month after the end of acaricide treatment, the mean daily mortality rate in the colonies that remained parasitized with *Nosema* spp. (group SYR) recorded during a one-month period was significantly higher than that in the *Nosema*-negative colonies (group FUM; *t*-test, $F = 9.95$, $P < 0.001$; Fig. 3C). Hence, since a strong correlation between natural mortality in colonies and *Varroa* infestation level has been proposed in some studies (Fries *et al*., 1991; Branco *et al*., 2006; Higes, 2008), it is suggested that these data may be reflecting significantly higher residual *Varroa* population after acaricide treatment in colonies that remained strongly parasitized by *Nosema* spp., involving a greater health risk for these colonies during overwintering. However, other methods, such as the application of a shock treatment with another acaricide to eliminate and collect all the remaining mites could have been more accurate to estimate mite population at this time point than the performed by means of natural *Varroa* mortality. The methodology used in this case was chosen for reasons of simplicity and efficacy (Fries *et al*., 1991) and to avoid chemicals overload in the colonies.

*Nosema* spp. are known to affect both the foraging behaviour and life span of bees, increasing the likelihood that foragers may not return to the colony (Higes *et al*., 2008; 2009; 2010b; Kralj and Fuchs, 2010). Similar findings have been reported in bees infested by *V. destructor* (Kralj and Fuchs, 2006), and according to this, the parasitized forager bees would die out of their colonies as has been reported for *N. ceranae* infected bees in field conditions (Higes *et al*., 2008), with the resulting decrease in the colony population size (Soroker *et al*., 2011). Moreover, the probability of foragers starving to death is also increased by their reduced capacity to fly back to the hive due to energetic stress in the case bees infected by *N. ceranae* (Mayack and Naug, 2009; Naug and Gibbs, 2009). One consequence of the high mortality rate of foragers is that uninfected bees may begin foraging at

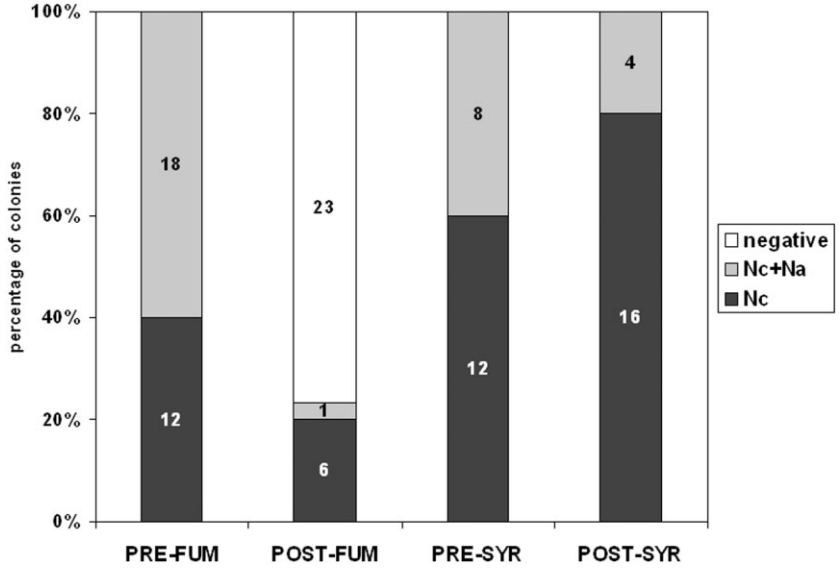


**Fig. 2.** Nosema infection data before and after interventions. In September, forager honey bees (n > 30) were collected at noon from the hive entrance of each of the experimental colonies to determine if the colonies had been naturally parasitized by *Nosema* spp. at this time point (Higes *et al*., 2008; Meana *et al*., 2010). The abdomens of 30 bees from each sample were macerated in 10 ml of ultrapure PCR grade water (Milli-Q system) and DNA extraction and PCR using an internal PCR control to determine the reliability of analysis were performed as previously described (Botías *et al*., 2011). Negative and positive controls were included in all the steps to detect any contamination. The same procedure was followed in the post-intervention period (November 2007).
A chi-squared test with exact probabilities was performed to test the null hypothesis of the independence of the response to treatment.
PRE-FUM and POST-FUM = Group FUM before and after fumagillin treatment respectively; PRE-SYR and POST-SYR = Group SYR before and after syrup administration respectively; Nc = *N. ceranae* infection; Nc + Na = *N. ceranae* and *N. apis* co-infection; negative = no detected.

younger ages to compensate for the loss of available foragers (Huang and Robinson, 1996; Amdam and Omholt, 2003; Khoury *et al*., 2011) and infected workers will also begin to forage prematurely and engage in other risky tasks at a precocious age (Tofilski, 2009). Since the division of labour in worker bees is age dependent, with young adults working within the hive on colony maintenance tasks and brood care, this premature change to foraging tasks may imply a disturbance over the colony social interactions and the longevity of individual bees (Khoury *et al*., 2011).

In *Nosema*-infected bees, this accelerated behavioural development has been linked with an increase in the titres of juvenile hormone (JH; Lin *et al*., 2009; reviewed by Higes *et al*., 2010b), the inhibition of vitellogenin (Vg) gene expression (Antúnez *et al*., 2009) and an increased level of the primer pheromone ethyl oleate (EO; Dussaubat *et al*., 2010). These three factors are each implicated in regulating the division of labour among worker bees, their maturation and the nurse-forager transition. In addition, several alterations in individual behaviour have been described in *N. ceranae*-infected honey bees (Mayack and Naug, 2009; Naug and Gibbs, 2009; Campbell *et al*., 2010), which could be associated with an indirect modulation of Vg levels by nutritional stress (Amdam and Omholt, 2003; Nelson *et al*., 2007) and may also contribute to increase the overall rate of foraging in the colony (Howard and Tschinkel, 1980; Toth *et al*., 2005).

These behavioural alterations at colony and individual honey bee level may have an impact on the effectiveness of acaricide strip treatment in field conditions. Indeed, these changes in behaviour may reduce the bees' contact with the strips and affect their interaction with other bees, thereby limiting the transfer of the active molecule throughout the colony.

In our experimental conditions, forager bees did not show *Nosema* infection after fumagillin treatment in 77% of treated colonies. Based on previous findings (Meana *et al*., 2010; M. Higes, unpubl. data), this decrease in infection assessed in foragers is likely to reflect a very low level or absence of infection in interior bees. Nevertheless, non-treated colonies (group SYR) remained parasitized by *Nosema* all through the assay. Thus, it is possible that the behavioural changes described in *Nosema*-infected colonies were more severe in these colonies, negatively affecting the effectiveness of the *Varroa* mite treatment.

Since prior to treatment with acaricide strips, comparable levels of *Varroa* mite parasite load were detected in the colonies studied, the differences observed in the mean daily *Varroa* mite mortality between groups following treatment appear to be associated with *Nosema* infection (see also Figs S3 and S4). Otherwise, as the *Nosema*

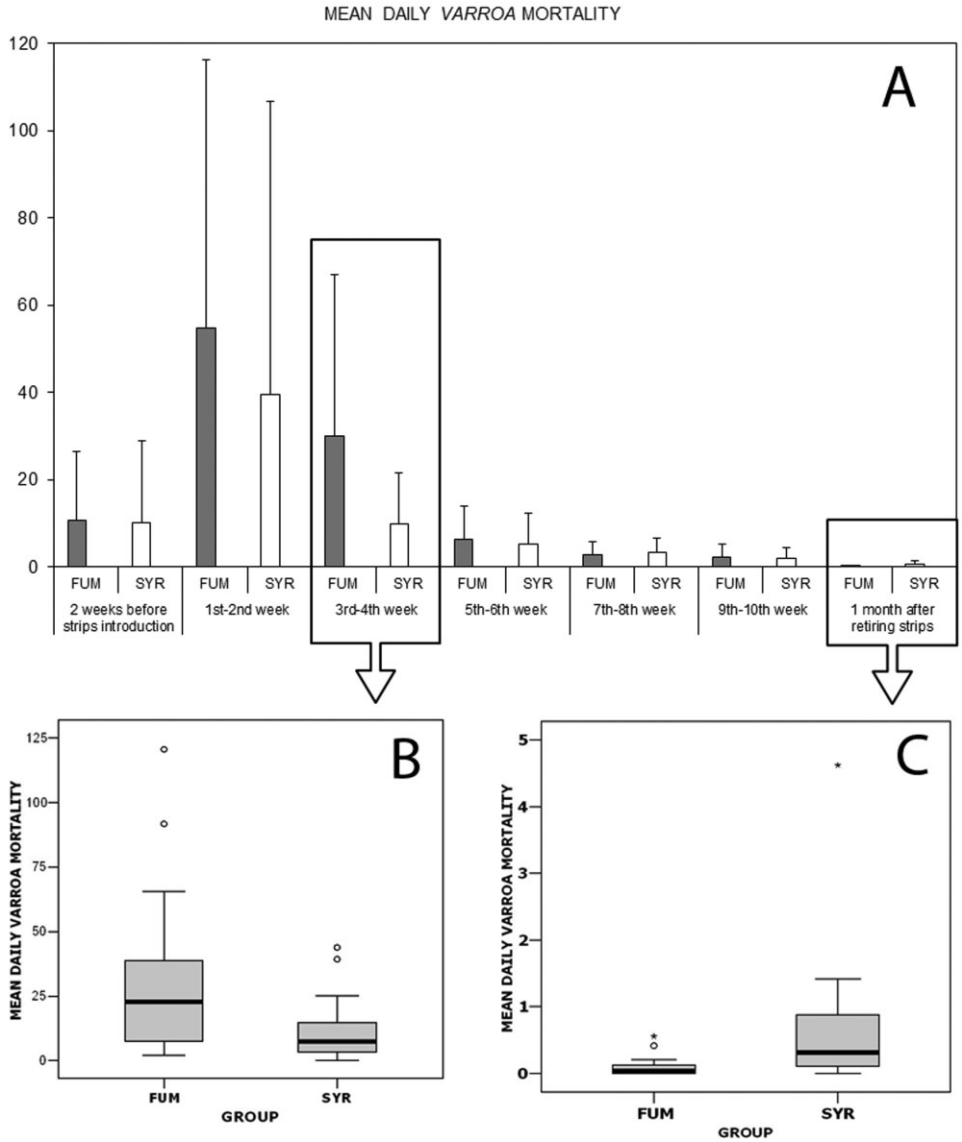


**Fig. 3.** Mean daily mortality of *Varroa* mites during the assay. Colonies were housed in modified Langstroth hives whose bottom boards were fitted with a 3 mm wire mesh in a wooden frame above a removable tray. Using the collection tray beneath the double floor board, fallen mites were gathered and counted at the laboratory.

All 50 experimental colonies were treated for *V. destructor* on the same day in October by placing two plastic strips of Apivar® (amitraz 0.5 g per strip) per colony between the frames close to the brood nest area and leaving them for 10 weeks in order to ensure proper treatment efficacy (Higes, 1999).

Mean daily mite mortality in each colony was determined following Guidelines on Veterinary medicinal products for the control of *V. destructor* parasitization of bees (European Medicines Agency, 2007). They were recorded in each of the following time points (see Fig. 1): (i) pretreatment natural mite mortality, during the 2 weeks prior to the start of the experiment; (ii) *Varroa* mortality induced by Apivar®, every two weeks after the initiation of treatment; and (iii) Post-treatment natural mite mortality, 1 month after removing the acaricide strips.

The comparison between the data of the *Varroa* mortality recorded in the two groups ($n = 50$ colonies; FUM = 30 and SYR = 20) was performed including all the colonies in the pretreatment assessment. However, for the other two periods (during treatment and post-treatment periods) those colonies of group FUM, which remained infected by *Nosema* spp. after fumagillin application ($n = 7$, see *Results and discussion*), were excluded from the comparison between groups ($n = 43$ colonies; FUM = 23 and SYR = 20) since the aim of the study was to compare the effectiveness of Apivar® between colonies infected by *Nosema* spp. versus non infected.

The means and standard deviations of the daily *Varroa* mite mortality were calculated in the two experimental groups at each of the time points indicated above. The records from the two groups were compared using the paired Student's *t*-test after Neperian log transformation of the data to normalize the distribution. Due to the different sizes of the two experimental groups, a non-parametric test (Mann–Whitney *U*-test) applying a Monte Carlo (two-tails) correction was used. The differences were considered statistically significant when $P \leq 0.05$.

A. Mean daily mortality of *Varroa* mites during the assay.

B. Box-plot of mean daily *Varroa* mortality during the third and fourth weeks of acaricide treatment.

C. Box-plot of mean daily *Varroa* natural mortality 1 month after retiring the acaricide strips.

infection was controlled by means of the administration of fumagillin and the mortality rate of *Varroa* mites tended to be higher in the case of fumagillin-treated colonies, a possible impact of this substance over the *Varroa* mites' health when feeding on the haemolymph of the treated bees may have occurred. Given that the possible acaricide effect of fumagillin has ever been reported, we consider that such interference over the present results is unlikely, but further research would be required to clarify this matter.

Earlier studies of asymptomatic and collapsed bee colonies in 2005, 2006 and 2007 described *N. ceranae* and *V. destructor* as the most prevalent pathogens in professional Spanish apiaries (Higes *et al*., 2010a; Bernal *et al*., 2011). The illnesses produced by these pathogens, nosemosis (mainly type C, as described in Higes *et al*., 2010b) and varroosis produce severe problems at the level of both the colony and the individual honey bee. Inadequate control of these pathogens can therefore be very detrimental to colonies and can significantly increase the risk of colony collapse, due to either pathogen alone or in combination with other synergistic factors (Higes *et al*., 2008; Bacandritsos *et al*., 2010; Bromenshenk *et al*., 2010; Rosenkranz *et al*., 2010; Whitaker *et al*., 2010).

Veterinary medicines such as Apivar® are presented as rigid or semi-rigid polymer strips impregnated with miticide. This acaricide was chosen for this study due to the dominant use of this product by Spanish beekeepers to control *Varroa* mites in recent years. The active ingredient is distributed throughout the colony after the bees come into contact with the strip and then subsequently contact other bees within the hive. As such, treatment efficacy is wholly dependent on these interactions. Our results suggest that this form of treatment is less effective in the colonies affected by nosemosis, probably due to the behavioural alterations induced by *Nosema* infection that may disturb colony homeostasis and organization, and alter foraging behaviour (Lin *et al*., 2009; Woyciechowski and Morón, 2009; Campbell *et al*., 2010; Dussaubat *et al*., 2010; Kralj and Fuchs, 2010), but this hypothesis should be further studied. This decreased effectiveness of *Varroa* mite treatment may have been attributed to tolerance of the *Varroa* mite to the acaricide, and a subsequent colony collapse solely linked to *Varroa* infestation, while *Nosema* infection may have triggered this phenomenon and in this case pathogens would have acted synergistically to affect the health of the colony.

Numerous colony losses have been reported over the last decade in Europe (COLOSS, 2009) and North America (vanEngelsdorp *et al*., 2008). Although the underlying causes remain unclear, most scientists agree that no single factor leads to colony decline but rather interactions between multiple stresses are probably involved (Le Conte *et al*., 2010). Co-infection of honey bees by multiple parasites has been well documented (Cox-Foster *et al*., 2007; Genersch *et al*., 2010; Higes *et al*., 2010a; Soroker *et al*., 2011), and several studies have reported synergistic interactions between parasites (Bailey *et al*., 1983; Downey *et al*., 2000; Downey and Winston, 2001; Bromenshenk *et al*., 2010). Considering the serious consequences for the colonies strongly parasitized by *V. destructor* (Kovac and Crailsheim, 1988; Murilhas, 2002; Boecking and Genersch, 2008) and *N. ceranae* (Higes *et al*., 2008; Hatjina *et al*., 2011; Soroker *et al*., 2011), and the negative effect of *Nosema* infection on *Varroa* control, more studies of the possible synergistic interactions between these pathogens in honey bee colonies are required. Also, future research tackling the use of other acaricides in colonies parasitized by *V. destructor* and *Nosema* spp. to compare with the present results would be of great interest and may determine the real effectiveness of each acaricide product in colonies parasitized by both pathogens.

Furthermore, the negative impact of the *Nosema* infection on *Varroa* mite treatment with acaricide strips should be considered when planning and assessing acaricide treatment strategies, particularly given the high prevalence of both pathogens worldwide.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Average percentage of *Nosema* spp. parasitization in the forager bees of the studied colonies before and after administration of vehicle (SYR, $n = 20$) or fumagillin (FUM, $n = 30$).
**Fig. S2.** Linear correlation between fumagillin consumption per colony (mg) and the percentage of *Nosema* infected bees (% parasitization) in the corresponding colonies of the group FUM.
**Fig. S3.** Mean adult bee population in each group during the assay.
**Fig. S4.** Mean number of the brood cells present in each group during the assay.

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SHORT COMMUNICATION

# Critical aspects of the *Nosema* spp. diagnostic sampling in honey bee (*Apis mellifera* L.) colonies

**Cristina Botías · Raquel Martín-Hernández · Aránzazu Meana · Mariano Higes**

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**Abstract** Nosemosis is one of the most widespread of the adult honey bee diseases and causes major economic losses to beekeepers. Two microsporidia have been described infecting honey bees worldwide, *Nosema apis* and *Nosema ceranae*, whose seasonality and pathology differ markedly. An increasing prevalence of microsporidian infections in honey bees has been observed worldwide during the last years. Because nosemosis has detrimental effects on both strength and productivity of the infected colonies, an accurate and reliable method to evaluate the presence of *Nosema* in honey bee colonies is needed. In this study a high degree of variability in the detection of microsporidia depending on the random subsample analyzed was found, suggesting that both sample size and the time of collection (month and day of sampling) notably affect the diagnosis.

C. Botías (✉) · R. Martín-Hernández · M. Higes
Laboratorio de Patología Apícola, Centro Apícola Regional (CAR),
Junta de Comunidades de Castilla La Mancha,
San Martín s/n. 19180,
Marchamalo, Guadalajara, Spain
e-mail: cbotias@jccm.es

R. Martín-Hernández
Instituto de Recursos Humanos para la Ciencia y Tecnología,
INCRECYT (FEDER funds), Parque Científico de Albacete,
Albacete, Spain

A. Meana
Departamento de Sanidad Animal, Facultad de Veterinaria,
Universidad Complutense de Madrid,
28040 Madrid, Spain

## Introduction

Nosemosis of honey bees is a highly prevalent and dangerous illness of bees worldwide (e.g., Farrar 1947; Weiser 1961; Moeller 1978; Liu 1984; Fries 1988). *Nosema apis* is one of the most important parasites of honey bees, and due to its lack of overt symptoms, it has been referred to as "the silent killer" (Hornitzky 2008). The colony losses resulting from infection by this parasite are thought to equal or exceed those caused by all of the other known bee diseases (Furgala and Musen 1990). Surprisingly, the effects *N. apis* infection on bee colonies have been overlooked largely by scientists and sanitary authorities, with few studies on this disease published prior to the start of this last century. Moreover, the World Organization for Animal Health (OIE) recently removed nosemosis from their list of notifiable diseases (OIE 2011a).

However, the last 5 years has seen an increase in the number of publications addressing the "*Nosema* problem," particularly since the discovery of *Nosema ceranae* infection in *Apis mellifera* (Higes et al. 2006; Huang et al. 2007) and its reported implication in the phenomenon of colony loss suffered in several countries (e.g., Higes et al. 2008, 2009; Bacandritsos et al. 2010; Borneck et al. 2010; Bromenshenk et al. 2010). *N. ceranae* is highly pathogenic in bees when acting alone (Antúnez et al. 2009; Higes et al. 2007; Paxton et al. 2007; Suwannapong et al. 2010) or in combination with other synergistic factors (Alaux et al. 2010), and it is more prevalent worldwide than *N. apis* (Klee et al. 2007; Martín-Hernández et al. 2007; Chen et al. 2009) with some exceptions (Giersch et al. 2009; Gisder et al. 2010). As such, *N. ceranae* infection may be the most prevalent and economically damaging disease affecting honey bees (Heintz et al. 2011) along with varroosis caused by the parasitic mite *Varroa destructor* (Le Conte et al. 2010; Rosenkranz et al. 2010). Due to the marked differences in the seasonality and pathology described

for both *Nosema* species (Paxton et al. 2007; Martín-Hernández et al. 2009; Chen and Huang 2010; Bourgeois et al. 2011), infection caused by *N. ceranae* has been recently proposed to be designated as nosemosis type C, which is a key emergent twenty-first century illness (Higes et al. 2010) whose sanitary status was recently reconsidered by the OIE (2010, 2011b). Given the economic and health impact of nosemosis type C in managed honey bee colonies, it is essential to optimize the sampling methods used to ensure a reliable diagnosis and an accurate analysis of the epidemiological evolution of this illness in field conditions.

In this study, we have evaluated the possible existing variability in the diagnosis of *Nosema* spp. in honey bee colonies depending on the subsample of honey bees analyzed and the time of collection (month and day of sampling).

## Materials and methods

Five naturally infected honey bee colonies located in our experimental apiaries were monitored at different days throughout the spring. The hive entrances were closed for 20 min at noon (Meana et al. 2010), and samples of at least 100 forager bees were collected upon their arrival at the corresponding hives. This sampling was performed on three consecutive days in May 2009, and it was repeated in June 2009. In our laboratory, honey bee samples from each colony were divided into five random subsamples of 20 bees that were analyzed separately by PCR as described below. The number of brood cells present in the brood combs of the colonies studied was also measured both in May and June by calculating the width and height of the brood area ellipses in each colony using a tape measure. The total number of brood cells (TBC) in each colony was determined by means of a standard ellipse formula (Higes et al. 1999), which assumes 4.12 cells per $cm^2$: $TBC=\sum(D\times d\times 0.785\times 4.12)$, where $D$ = longest diameter of the ellipse, $d$ = smallest diameter of the ellipse, and 0.785 is the coefficient. The average TBC value was calculated for each of the five colonies at each time point, and the means were compared by Student's $t$ test.

To detect *Nosema* spp., each bee subsample ($n$=20 bees) was macerated for 2 min at high speed in 10 ml $H_2O$ PCR grade (PCRq) in a Stomacher® 80 Biomaster (Seward) using filtered bags (BA6040/STR, Seward). The filtered macerate was recovered in a tube, and subsequently, 10 ml of $H_2O$ (PCRq) was added to the stomacher bag and the maceration process was repeated as described above. Macerates were then centrifuged at 514×$g$ for 10 min, the supernatant was discarded, and the pellet was resuspended in 1 ml of ultrapure water (PCRq). This final suspension was shaken 30 times per s for 4 min with 0.1 g of glass beads (2 mm diameter) in a TissueLyser (Qiagen). Subsequently, 150 μl of the homogenate was incubated overnight at 56°C with 20 μl of proteinase K and 30 μl of ATL buffer (Qiagen), and DNA was extracted using a BioSprint™ 96 DNA Blood Kit (384: Qiagen, Cat. No. 940057) in a Biosprint 96 robot (tissue protocol Qiagen). The DNA extracts were analyzed by duplex PCR as described by Martín-Hernández et al. (2007) using a Mastercycler ep gradient S (Eppendorf), and each PCR product was analyzed in a QIAxcel System (Qiagen), using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002). Duplicate reactions were performed to confirm the results. In addition, DNA extracts from every five subsamples belonging to the same initial sample were pooled in order to evaluate consistency of these results with those obtained when analyzing individual subsamples. To do so, 20 μl of the DNA extracts from the five subsamples were combined as one unique homogenate, getting a final volume of 100 μl of DNA extract. This homogenate was analyzed by PCR as described above.

## Results and discussion

A high level of variability was observed between individual subsamples from the same initial sample, and in some cases, different results were obtained from the same hive depending on the day of sampling (Fig. 1), primarily in samples collected in May. Thus, it was common to observe colonies that tested negative or *N. ceranae* positive just depending on the subsample analyzed (e.g., colonies BN9, BN7, BN12, B2), or even negative and parasitized by both microsporidia (colony BN8, BN9). Surprisingly, in some cases (colony BN8 sampled on 23 June 2009), all possible outcomes were observed (i.e., negative and positive for both *N. apis* and *N. ceranae* in single and mixed infections). The results of the samples collected in June were less variable, in which *N. ceranae* was the predominant microsporidium detected in the different subsamples. The average number of brood cells present in the brood chamber in May (23,884±5,329) was significantly higher than that found in June (20,123±5,990; Student's $t$ test, $P<0.001$), suggesting a greater number of newly emerged bees in the colonies in May.

These results reveal the high degree of variability in the detection of microsporidia depending on the random subsample analyzed, even when following OIE-recommended (2008) sampling procedures. When five different subsamples of 20 bees from the initial samples collected on three consecutive days in May and June were analyzed, a completely different diagnosis was obtained in several colonies depending on both the day of sampling and the random subsample analyzed. These findings suggest that both sample size and the time of collection notably affect the diagnosis, and thus, the analysis of a single subsample collected at a specific time point cannot provide conclusive information about the presence of *Nosema* spp. in the colony or apiary, particularly in the spring months.

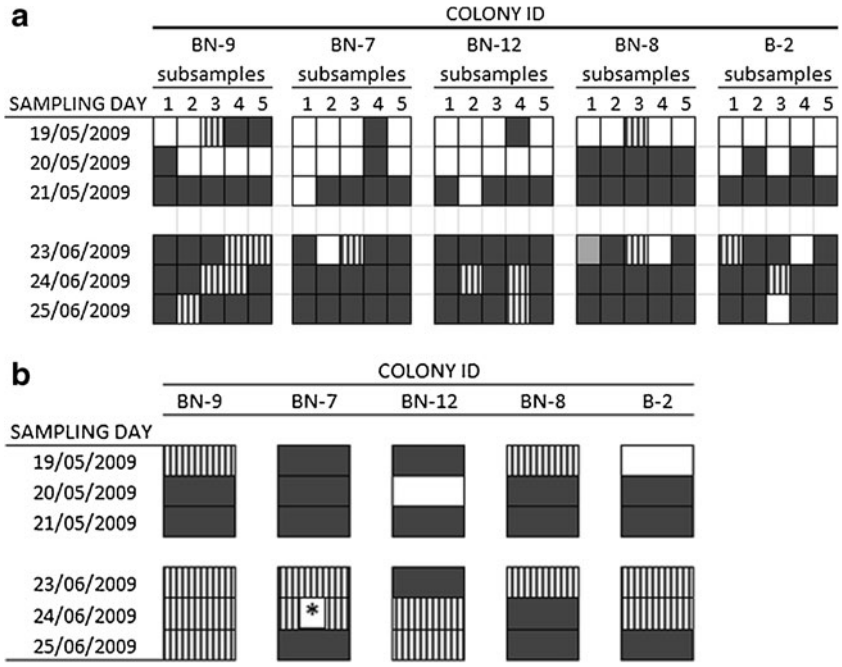


**Fig. 1** **a** PCR results from the five random subsamples ($n$=20 bees) collected from experimental colonies on each sampling day (*dark grey N. ceranae* positive; *light grey N. apis* positive; *vertical strips* coinfection with *N. ceranae* and *N. apis*; *white Nosema* spp. negative). **b** PCR results from the pooled DNA extract resultant from gathering the DNA extracts from every five subsamples belonging to the same initial sample (*dark grey N. ceranae* positive; *vertical strips* coinfection with *N. ceranae* and *N. apis*; *white Nosema* spp. negative; *asterisk* indicates inconsistent result regarding individual subsamples analysis)

Samples larger than 20 bees would probably provide more homogeneous results and consequently a more reliable diagnosis of *Nosema*. When the five subsamples separated from the same initial sample were combined, we found consistent results in all of the cases, except for one of the pooled DNA extracts (colony BN-7 on 27 June 2009; Fig. 1b). In this case, the combined extract tested positive to *N. apis* and *N. ceranae*, whereas all of the subsamples separately analyzed only tested positive to *N. ceranae*, being the signal for *N. apis* in the electrophoresis of the PCR product from the pooled DNA extract very low. This inconsistency may be explained as a stochastic effect in the PCR associated to a reduced presence of *N. apis* in this colony, as has been previously reported when a low copy number of DNA is present in a template (Taberlet et al. 1996). In fact, in the previous day of sampling (23 June 2009), this microsporidium was detected in one of the subsamples analyzed. This event confirms the need for bigger sample sizes and frequent samplings, since when the parasitic load of a specific pathogen is low, it is hardly detected, and an early detection may be of high importance to take the necessary measures to reduce morbidity and mortality.

The diagnostic analysis of *Nosema* spp. is usually performed using a random fraction (10–20 bees) taken from the sample collected from the colony. This approach may significantly influence the results obtained in a diagnostic trial and any conclusions drawn from these results, which is an issue that should be taken into account when making subsequent comparisons.

In conclusion, as the sample size and the time of collection (month and day of sampling) affect diagnostic results, larger sample sizes and regular and frequent sampling are recommended to accurately determine honey bee infection by *Nosema* spp. in managed honeybee colonies. This may be particularly important in the spring months, when more newborn bees are present and thus the proportion of infected individuals in the colony may decrease. The observed differences in diagnostic results between months may also be linked to differences in the development of microsporidia infection in the honey bee colony throughout the year (Pickard and El-Shemy 1989; Higes et al. 2008), and maybe, a different sample size would be necessary depending on the season and/or the climatic conditions under which the corresponding honey bee colonies are being evaluated. Further research performing a similar study under different weather conditions and different sample sizes would be of great interest to confirm the high variability of results in the diagnosis of *Nosema* spp. found in the present assay.

The ideal number of bees to be collected in a sample cannot be ascertained based on our results, and more studies will be necessary to clarify this issue. However, a recent study using *Nosema* spore counts to diagnose levels of infection by *N. ceranae* recommended samples of at least 50 bees (Bourgeois et al. 2011).

The findings presented here should be taken into consideration when planning epidemiological studies or diagnostic laboratory tests of infection by *Nosema* spp. A standardization of the diagnostic sampling method to detect *Nosema* spp. is necessary to guarantee reliable and comparable results.

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La abeja melífera es un insecto esencial para la conservación de la biodiversidad en nuestro planeta debido a su acción polinizadora tanto de plantas silvestres como cultivadas, además de enriquecer nuestra dieta con productos propios como la miel, la jalea real o el polen apícola. Existen una serie de factores que condicionan la viabilidad de la abeja melífera como especie productora y polinizadora, y entre ellos se encuentra la acción patógena de los microsporidios *Nosema apis* y *Nosema ceranae*. El control y la prevención de las nosemosis, apoyados en un conocimiento del patrón epidemiológico de estas enfermedades, supondrá un gran beneficio para estos valiosos insectos sociales y por extensión, tanto para la humanidad como para la biodiversidad de nuestro planeta.